SYMPOSIA

Newer Methods For The Diagnosis of Childhood Tuberculosis

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For an infectious disease like tuberculosis, which is transmitted by aerosol droplets, the rapid and accurate detection of *M.tuberculosis* is essential, not only to speed up the treatment of the patient but also to control the spread of the disease.

Tuberculosis in childhood occurs with different manifestations. All these forms of tuberculosis, except when cavitation occurs in pulmonary tuberculosis, are paucibacillary in nature. For this reason, even though at the present time bacteriological confirmation is still the final proof of tuberculous disease, it is difficult to obtain.

Depending on the form of disease manifestation, several specimens like sputum and/or gastric lavage, as children are often unable to produce sputum, lymph nodes and other biopsy specimens, pus, ascitic fluid, pleural or cerebrospinal fluid (CSF) need to be collected. If delay is anticipated, relevant specimens may be collected in suitable transport medium for sending it to the laboratory.

There are two ways to address diagnosis of tuberculosis. The direct approach is concerned with the detection of the bacteria by microscopy or culture, detection of tuberculostearic acid (bacterial wall component), detection and identification of mycobacterial antigen by the use of polyclonal or monoclonal antibodies, analysis of lipid composition by chromatography, and the detection of DNA or RNA of mycobacterial origin by hybridization with a DNA probe with or without amplification of nucleic acids. The indirect approach relates to measurement of host immune response against the mycobacteria. This includes humoral immunity via the detection of antibodies against the bacteria and cellular response via skin tests.

TRADITIONAL APPROACH

1. Microscopy

Microscopy is the simplest and most rapid procedure currently available to detect acid-fast bacilli (AFB) in clinical specimens by Ziehl Neelsen or Kinyoun staining procedure. The limit of detection with this method is 5000-10,000 bacteria per ml of sputum. Introduction of fluorochrome dyes such as Auramine O in place of the traditional basic fuchsin dyes made smear reading advantageous as the yellow-fluorescing bacilli are easier to detect than the red fuchsin-stained organisms seen in Ziehl-neelsen or Kinyoun staining. Further, in the fluorescent microscopy, smears are read in low power so that larger areas are screened in less time. The results of smear microscopy can be influenced by many factors such as the type of counter stain used, and the training and experience of the person evaluating the smear. A new approach to increase the smear sensitivity is to use cytocentrifuges[1]. The main advantage of sputum smear microscopy is that it is inexpensive, well-established, extremely reliable and results can be reported within hours of receipt of the sample. However, viability of the organism and species identification are not possible with this technique.

II. Culture and Drug Susceptibility

Isolation of mycobacteria from clinical specimens has solely depended on the use of media such as the egg-based Lowenstein- Jensen (LJ) medium, Middlebrook 7H11 agar, Middlebrook 7 H9 broth and Kirchner's liquid medium. The major constraint of this procedure is the slow growth of mycobacteria with necessitates a mean incubation period of 4 weeks on these

conventional media. The drug susceptibility tests to the antituberculosis drugs require additional 4 weeks before results can be obtained.

MODERN APPROACHES

Today, there are a few rapid methods for the culture of mycobacteria. These include microcolony detection on solid media, the Septi-chek AFB method, mycobacterial growth indicator tuber (MGIT) system, radiometric Bactec 460 TB method and Bactec MGIT 960 method.

I. Microcolony counting method

In this method, plates poured with thin layer of Middlebrook 7H11 agar medium are inoculated and examined microscopically on alternate days for the first 2 weeks and less frequently thereafter. For microscopic examination, the plates are inverted on the stage of a conventional microscope and the objective is focused on the surface of the agar. In less than 7 days, microcolonies of slow growing mycobacteria such as *M.tuberculosis* can be detected using this method. Though this method is less expensive and requires about half the time needed for conventional culture, the recovery of mycobacteria is less efficient and it is labour intensive[2]. However, sensitivity of sputum microscopy is limited in detecting AFB in extra-pulmonary specimens especially in pauciba-cillary specimens such as gastric lavage, bron-chioalveolar lavage, CSF and urine samples etc.

II. Septi-check AFB system

It consists of a liquid phase 7H9 broth with three solid media, the Middlebrook 7H11 agar, modified egg medium and chocolate agar. This biphasic medium is presented in a self contained CO_2 environment. This non-radiometric approach has the potential to expedite processing, obviate CO_2 incubation requirements and facilitates early detection of positive cultures. This method requires about three weeks of incubation. The unique advantage of this technique is the simultaneous detection of M.tuberculosis, non-tuberculous mycobacteria (NTM), other respiratory pathogens and even contaminants. It has been reported from multicentric studies conducted by different groups that this system gives a better culture result when compared to other methods including BACTEC[3].

III.Mycobacterial growth indicator tuber (MGIT) system

It is a non-radiometric broth method for the growth and detection of mycobacteria from clinical specimens. The MGIT system consists of a Middlebrook 7H9 broth and a fluorescent compound embedded in silicone. Antibacterial, antifungal agents and growth supplements are added before inoculation. Positive cultures are detected visually because of the metabolic depletion of oxygen, which otherwise quenches fluorescence. Instrumen-tation is not required, and multiple tubes can be inspected simultane-ously. The tube is examined under UV light where the growth is indicated by an unmistak-able orange fluorescent glow. In several studies conducted elsewhere, it was found that the culture sensitivity of this method in both smear positive and smear negative samples compared well with the BACTEC 460 and it was better than the conventional egg based method [4].

IV. Radiometric BACTEC 460 TB method

Middlebrook *et al* developed this technique which is specific for mycobacterial growth, wherein C^{14} labelled palmitic acid in 7H12 medium is used. This system detects the presence of mycobacteria based on their metabolism rather than their visible growth. When the ^{14}C-labelled substrate present in the medium is metabolized, $^{14}CO_2$ is produced and measured by the BACTEC 460 instrument and reported in terms of growth index (GI) value. In addition to detecting mycobacteria, BACTEC system is also useful in the identification of *M.tuberculosis* using the specific inhibitor p-nitro-alpha-acetylamino-betahydroxy propiophenone (NAP). The NAP differential procedure has been shown to be a rapid and reliable method for identifying *M.tuberculosis*. Using the same system, drug susceptibility tests can be performed for all the antituberculosis drugs when sufficient growth index (GI) is observed. Mycobacteria in clinical samples can be detected in half the time that is needed for conventional culture methods.

A comparison of the BACTEC radiometric method with the conventional culture and drug susceptibility testing methods on isolated from clinical specimens was recently published from Tuberculosis Research Centre, Chennai. The results showed that the rate of isolation of posi-

> "Ligase chain reaction system has been developed which can further improve the confirmation of *M. tuberculosis* growth by BACTEC system even when there is low growth index"
> Tostoli E, et al
> J Clin Microbiol
> 1998; 36: 2791 -2.

> "Sensitivity of sputum microscopy is limited in detecting AFB in extrapulmonary specimens such as gastric lavage, bronchioalveolar lavage, CSF and urine".

tive cultures was significantly faster with the BACTEC method, with 87% of the positives being obtained by 7 days, and 96% by 14 days. There was a good correlation in drug susceptibility tests and most of these results became available within 8 days by the BACTEC methods. By facilitating early diagnosis, the BACTEC method may prove to be cost effective in a population with a high prevalence of tuberculosis [3.5].

V. BACTEC ® MGIT 960 mycobacteria detection system

It is an automated system for the growth and detection of mycobacteria with a capacity to incubate and continuously monitor 960 MGIT culture tubers. The culture tuber contains same Middlebrook 7H9 broth base. OADC enrichment and PANTA antibiotic mixture as does the manual MGIT, except that the final volume here is 7ml. After adding 0.5 ml inoculum of the processed specimen, the tuber are incubated at 37°C in the BACTEC MGIT 960 instrument, and are monitored automatically every 60 minutes for increase in fluorescence. Growth detection is based on the AFB metabolic O_2 utilization and subsequent intensification of an O_2 quenched fluorescent dye contained in a tube of modified MGIT. A series of algorithms are used to determine presumptive positivity and alert the operator to the presence and location of positive tubes.

In a multi-centre evaluation of the BACTEC MGIT 960 system, three high-volume testing sites in USA compared the growth and recovery of AFB to that of the BACTEC 460 TB and conventional culture. Comparison of average time of detection between paired specimens showed that, the BACTEC 460 TB and BACTEC MGIT 960 systems were 8.7 versus 8.6 for MAC and 13.4 versus 15.5 days for *M.tuberculosis* respectively. According to these investigators the BACTEC MGIT 960 system exhibits greater potential as a rapid, accurate and cost effective method for a high volume AFB laboratory[6].

SPECIES IDENTIFICATION

The conventional identification procedures include tests such as determination of growth rate, growth at different temperatures, colony morphology, pigment production and susceptibility to para-nitrobenzoic acid (PNB). The main disadvantage here is that species identification takes

an additional 4 weeks using these methods.

NEWER METHODS FOR SPECIES IDENTIFICATION

I. High performance liquid chromatography (HPLC)

In this technique, the pattern of long chain high molecular weight fatty acids unique to mycobacteria and related genera, namely the mycolic acids, is generated and compared using an automated chromatograph after extraction of the lipid component of the mycobacterial cells using standardized protocols. These patterns have been found to be species-specific thus allowing rapid identification of mycobacterial cultures. Software based on mycolic acid patterns for correct identification of the most commonly encountered mycobacterial species in the clinical laboratory is now commercially available. This method is reliable, easy to perform and less expensive than DNA based identification methods such as DNA probes and PCR. However, this technique requires considerable expertise[7].

II. DNA probes

The nucleic acid base-pairing is intrinsically very specific and nucleic acid probes have been developed which are capable of recognizing specific portions of DNA or RNA. The choice of the target DNA sequence for a DNA probe determines the specificity and sensitivity of the test. The probe can consist of a fragment of the mycobacterial genome, a cloned DNA fragment or a synthetic oligonucleotide. The nature of the probe influences its specificity. The sensitivity of the probe depends on the detection system and the copy number of the target sequence in the genome. Usually better results are obtained if the probe is targeted to sequences present in multiple copies in the target cell (in this case the mycobacterium) or if the target sequence is present in the form of repetitive DNA or robosomal RNA. Commercially available DNA probes hybridize to species-specific rRNA sequences of *M.tuberculosis*, *M.avium-intracellulare, M.kansaii* or *M. gordonae.* The mycobacterial probes developed by Gen-Probe (San Diego, CA) were originally labelled with ^{125}I and required physiological separation of hybridized and unhybridized probe before detection in the gamma counter. Now the commercial probes use a

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chemiluminescent (non-radioactive) label and the hybridized and unhybridized probe are separated by adding a chemical 'selection' agent. The chemiluminescent procedure requires a nominal time of 45 minutes and the acridinium ester label is more stable and extends the shelf life of the probe reagent to about 6 months. However, these probes still require about $1\text{-}3 \times 10^7$ bacilli for a positive test result while traditional culture techniques require about 10^{-2} bacilli/ml of specimen. This indicates that RNA as a target may not provide the required sensitivity of direct detection of mycobacteria in clinical samples. Another method using solid phase (nylon membrane) non radioactive DNA hybridization has been developed for identifying *M.tuberculosis* and *M.avium-intracellulare* by Sygene Inc. (San Diego, CA). This method uses oligonucleotide probes with an alkaline phosphatase label and hybridization of the probe to the target is detected with a chromogenic substrate (reduction of nitroblue tetrazolium)[8].

III. Nucleic acid amplification

Nucleic acid (DNA or RNA) amplification techniques can detect and identify mycobacteria directly in clinical samples. The most commonly used method is the polymerase chain reaction (PCR). In this technique, target DNA sequences are exponentially amplify in repeated cycles of DNA synthesis driven by specific oligonucleotide primers. The amplified DNA is usually detected by agarose gel electrophoresis. Today, many PCR assays are available for the detection of M.tuberculosis complex in clinical samples. The most common of these is the one using the insertion sequence IS6110 which is present only in mycobacteria belonging to the *M.tuberculosis* complex. Like microscopy, PCR results can be obtained within 24 hours of receipt of the sample. It is, however, important to remember that sensitivity and rate of correct identification varies widely between different laboratories.

The current commercially available amplification tests are expensive and do not have any control for inhibitors of amplification. This may produce false-negative reactions. The main advantage of this technique over conventional microscopy and culture is the speed with which the diagnosis can be made and direct identification of the infecting mycobacterium.

A recent modification of nucleic acid amplification is the strand displacement amplification (SDA) method which is an isothermal, in vitro amplification of nucleic acids. The system has been designed to amplify either the 16S rRNA gene sequence for mycobacteria or the insertion element IS6110 for *M.tuberculosis.* The main advantage of this method is that it works at a single temperature which eliminates the need for expensive thermal cyclers required by the PCR method. Additionally, it is not susceptible to contaminating ribonuclease activity which is a major problem in clinical samples. However, isothermal techniques require additional restriction enzymes which may increase the overall cost of the procedure. It should also be borne in mind that this procedure is still being evaluated[19,10].

MOLECULAR BASIS OF DRUG RESISTANCE

Since multi drug resistant tuberculosis strains (i.e. resistance to at least rifampicin and isoniazid) are rapidly emerging and further burdening the already considerable global tuberculosis problem, it is becoming imperative to rapidly diagnose drug resistant *M. tuberculosis* strains[11].

In contrast to other bacteria, drug resistance in *M.tuberculosis* is not plasmid mediated. Although molecular events that are responsible for drug resistance have not been well defined in all cases, recent studies have shown a tremendous progress. INH resistant mutants of *M.tuberculosis* strains frequently showed catalase negative phenotype which could be attributed to deletion or some point mutation in Kat G gene[12,13]. Recently, resistance to rifampicin was found to be determined by mutations causing change in the rpo B gene coding for beta-sub unit of RNA polymerase[14].

Streptomycin-resistant mutants show alterations in the ribosomal protein S12 and/or in the highly conserved rspl gene, which encodes the S12 ribosomal protein[15]. The single amidase enzyme with both pyrazinamidase (PZase) and nicotinamidase activities in *M.tuberculosis* is the target for pyrazinamide. The mutations in pnc A gene encoding the amidase enzyme confers resistance to pyrazinamide in *M.tuberculosis* complex[16]. Quinolones and coumarins are the known inhibitors of the DNA gyrase. The gyrA

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mutations have been found to be associated with resistance to quinolones[17].

Techniques such as single strand cellular polymorphism (SSCP) could identify mutations in short piece of DNA. DNA of interest could be amplified by the Polymerase Chain Reaction (PCR). The PCR-SSCP technique has been successfully explored for the detection of mutations in the rifampicin resistance and also resistance for streptomycin and quinolones[18].

A new technique called the Line Probe Assay (LiPA) also has been evolved using PCR to amplify the region of RNA polymerase involved in rigampicin resistance. A set of oligonucleotide probes are immobilised onto a membrance strip and rifampicin resistnce can be assessed from the pattern obtained following hybridization. This technique is undergoing rigorous analysis at the moment[19].

Phage reporter assay

In this technique, viable mycobacteria are infected with specific reporter phages expressing firefly luciferase gene Easily detectable signals are apparent a few minutes after infection of *M.tuberculosis* with reporter phages. Light production requires metabolically active *M.tuberculosis* cells, in which reporter phages replicate and luciferase gene is expressed. When drug-susceptible *M.tuberculosis* strains are incubated with specific anti-tuberculosis drugs, they fail to produce light after infection with luciferase reporter phages. In contrast, drug-resistant strains are unaffected by the drugs and produce light at levels equivalent to those documented for untreated controls after infection with reporter phages. This procedure may reduce the time required for susceptibility testing of *M.tuberculosis* strains. The applicability of this technique to drug susceptibility testing of non-tuberculous mycobacteria is yet to be explored[20,21]. An evaluation of a new rapid bacteriophage based method for the drug susceptibility testing of *M.tuberculosis* has been reported recently by Wilson *et al* by the Pha B assay. The concept of this assay is based on the ability of viable *M.tuberculosis* bacilli to protect infecting mycobacteriophage from inactivation by phagocidal chemicals. It follows that after incubation with drugs only those *M.tuberculosis* that

remain viable (i.e are drug resistant) can protect the mycobacteriophage. Any mycobacteriophage protected within viable bacilli replicate and ultimately lyse their host. For rapid detection, the released mycobacteriophage are mixed with a rapidly growing *M.smegmatis* host in which they undergo rapid cycle of infection, replication and lysis. Lysis is easily seen as clear areas or plaques in a lawn of rapid grower host. The number of plaques generated from a given sample is directly related to the number of protected mycobacteriophage, which is dependent on the number of *M.tuberculosis* bacilli that remain viable after drug treatment[21].

SKIN TESTS

Delayed type hypersensitivity (DTH) reaction followed intradermal injection of a predetermined amount of tuberculin or purified protein derivative (PPD) remains the simplest and frequently used method of inferring tuberculous infection. Although skin test does not discriminate between active tuberculosis, post infection with mycobacteria or BCG vaccination, in the diagnosis of tuberculosis it plays an important role especially in association with contact history[22].

ANTIBODY DETECTION

In this technique, antibodies against mycobacterial antigens in sera from patients by Enzyme-Linked Immunosorbent Assay (ELISA) is detected using polyclonal or monoclonal antibodies. When crude mycobac-terial preparation are used for these tests, the test has low specificity. False-positive results are likely to be due to cross-reactions by environ-mental mycobacteria and other bacteria. To prepare purified antigens from mycobacterial cultures, techniques such as ion-exchange chromatography, gel filtration, isoelectric focusing, electrophoresis and affinity chroma-tography are required, purified antigens improve the specificity of the antibody detection assays. However, reproducible methods for the purification of mycobacterial antigens have yet to be evolved which means that the results of such assays are variable. The most promising purified antigen among those available so far are the 38kDa and Ag60 antigens from *M.tuberculosis*[23]. At this point it is worthwhile to mention that the immune response in mycobacterial disease appears to be associated with HLA class type II

allotypes and different patients appear to recognize different antigens[24]. This suggests that it is unlikely that all tuberculosis patients will recognize a single antigen. This may prove to be a handicap as far at antibody-based detection systems for mycobacteria are concerned[25].

CONCLUSIONS

Today, many new techniques are available for the diagnosis of tuberculosis and also for detection and identification of mycobacteria. However, detection of AFB by direct microscopy and identification of cultured mycobacteria by biochemical methods still remain the recommended methods. Since about a third of children with tuberculosis suffer from extrapulmonary disease, it is necessary to culture all fluids and biopsy specimens from appropriate body sites, where the conventional methods may offer limited help. Other non-specific tests such as white blood cell count, differential count, sedimentation rate, lysozyme assay, bromide-partition test, adenosine deaminase assay and estimation of sugar and protein may play a supportive role. Faster culture methods such as Bactec, MGIT are being increasingly used mainly because they reduce the time of culture to about 2 weeks. Nucleic acid amplification techniques are mainly for cases where there is a change that the infection may be due to a mycobacterium other than *M.tuberculosis*. It is also to be remembered that most of the new techniques described involve prohibitive expenditure in terms of instrumentation, expertise and reagents, putting them out of the reach of many public health laboratories of developing countries, especially India[26].

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