Tuberculosis: Epidemiology and Diagnosis

C.N. Paramasivan
Bacteriology Division
Tuberculosis Research Centre (ICMR)
Chennai 600 031, India

Despite the discovery of the tubercle bacillus more than a hundred years ago, and all the advances in our knowledge of the disease since then, tuberculosis still remains one of the major health problems facing mankind, particularly in developing countries. About one third of the World's population is infected with *M. tuberculosis.* It is estimated that currently there are about 9 million new cases of tuberculosis with 3 million deaths worldwide. More people die of tuberculosis than any other infectious disease. Death from tuberculosis comprises 25% of all avoidable deaths in developing countries. Ninety five per cent of tuberculosis cases and 98% of tuberculosis deaths are in developing countries and 75% of tuberculosis cases are in the economically productive age group[1]. Geographically, the regions with the highest prevalence and infection rates are the eastern fringe of Asia, the Indian subcontinent, the South eastern part of Africa, South-east Europe, Central America and the Western part of the South America. The WHO has declared a global emergency in 1993 with respect to reemerging menace of tuberculosis[2].

Epidemiology of tuberculosis in India

In India, out of a total population of approximately 950 million people, each year over 200 million develop active disease and upto 500,000 people die which roughly accounts for one sixth of the 3 million deaths occurring due to tuberculosis globally. It implies that every minute, a death occurs due to tuberculosis in our country[2]. It also imposes a cost on economy in terms of current and future output loss because of premature death and ill health[3].

HIV and tuberculosis

HIV has a serious effect on tuberculosis in many parts of the world. Tuberculosis is considered as one of the most common human immunodeficiency virus (HIV) related opportunistic infections. The frequency with which HIV and *M. tuberculosis* infection occur together is determined by the epidemiology of each disease in a given population. The epidemic of HIV associated tuberculosis has been well documented in many places

including sub-Saharan Africa and United States of America. WHO's global tuberculosis programme has estimated that the total number of new tuberculosis cases attributable to HIV will rise from 3 lakh in 1990 to around 1.4 million by the year 2000. The percentage of tuberculosis cases due to HIV infection will thus rise from 4% to 14% of the total number of cases. The annual risk of breaking down with tuberculous disease among dually infected persons is around 10% per year compared to 10% life time risk among those infected with tuberculosis alone. Approximately 6 million people are currently co-infected with tuberculosis and HIV worldwide[4]. Although accurate estimates are not available for India, available reports indicate that the prevalence of HIV in tuberculosis patients is increasing all over the country from 2% to 24%. With the spread of HIV epidemic, several countries have observed a rapid increase in the incidence of tuberculosis. The mortality and morbidity caused by the combined onslaught of the dual infection is quite alarming[5]. The level of initial drug resistance is said to be an epidemiological indicator to assess the amount of resistant bacterial transmission in the community. The prevalence of drug resistant tuberculosis varies considerably throughout the world. The reasons for this are selection of patients studied, misuse of drugs and the quality of enquiry regarding previous treatment etc. A decline in the percentage of drug resistance in tuberculosis was observed in Korea and New York city in recent years. But in India, the percentages have remained static or increased and over the years the initial resistance to isoniazid as single agent is shown to range from 0.6-13.2%, streptomycin from 2.2-7.0% and rifampicin from 0-1.7%[6].

The prevalence of MDR TB in India is low in most of the regions and ranges from 0-6%. Primary MDR TB is found to be <3.2% and the level of acquired MDR-TB is <6.0% except in Gujarat (14-18.5%) and North Arcot, Tamil Nadu (20.3%) and in new Delhi (13.3%) where a high level was reported. When compared to the prevalence of MDR-TB in other parts of the world where upto 48% have been encountered, lower levels of resistance are evident from Indian studies.

Laboratory diagnosis

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 in the population.

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 where larger areas are screened in less time.

The results of smear microscopy can be influenced by many factors such as the type of specimen, the mycobacterial species, decontamination methods used, the thickness of the smear, the extent of decolorization, 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[7]. 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 7H9 broth and Kirchner's liquid medium. The major constraint of this procedure is the slow growth of mycobacteria which 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 tube (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 incubated 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 focussed 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[8].

II. Septi-Chek AFB system

It consists of a liquid phase 7H9 broth with three solid media the middle brook 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 multi-centric studies conducted by different groups that this system gives a better culture result when compared to other methods including BACTEC[9].

III. Mycobacterial growth indicator tube (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. Instrumentation is not required, and multiple tubes can be inspected simultaneously. The tube is examined under UV light where the growth is indicated by an unmistakable 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[10].

IV. Radiometric BACTEC 460 TB method

This technique 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-beta-hydroxypropiophenone (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 isolates from clinical specimens was undertaken at our Centre. The results showed that the rate of isolation of positive culture 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 correction in drug susceptibility tests and most of these results became available within 8 days by the BACTEC method. By facilitating early diagnosis, the BACTEC method may prove to be cost effective in a population with a high prevalence of tuberculosis[9,11].

V. BACTEC(R) 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 tubes. The culture tube contains same Middlebrook 7H9 broth base, OADC enrichment and PANTA antibiotic mixture as does the manual MGIT, except the final volume here being 7 ml. After adding 0.5 ml inoculum of the processed specimen, the tubes 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[12].

Species identification

The conventional 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.

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[13].

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 ribosomal RNA. Commercially available DNA probes hybridize to species-specific rRNA sequences of *M. tuberculosis, M. avium, M. intracellulare, M. kansasii* 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 unhybridised probe before detection in the gamma counter. Now the commercial probes use a chemiluminescent (nonradioactive) label and the hybridized and unhybridised 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 1-3x10^7 bacilli for a positive test result while traditional culture techniques require about 10^2 bacilli/ml or sputum. 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)[14].

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. There is, therefore, a need to develop an effective system to carefully monitor the sensitivity since clinical samples may contain DNA polymerase inhibitors. To control such inhibition, it is now common practice to add a small amount of *M. tuberculosis* DNA to the sample. However, this step considerably increases the number of PCR assays necessary for each clinical sample. The latest development in this regard is the construction of a modified *M. smegmatis* strain with a modified IS6110 fragment integrated into the genome which can serve as an efficient internal control to monitor the efficacy of DNA extraction and to detect the presence of PCR inhibitors. It is also absolutely necessary to evolve a system to prevent contamination

with DNA fragments. The most frequent cause of false-positive results is contamination with minute amounts of amplified DNA fragments (amplicons) *via* aerosols spread by laboratory workers during handling of the PCR products. Contamination with one molecule is sufficient for a false-positive PCR result. The use of uracil DNA glycosylase in combination with dUTP instead of dUTP can efficiently prevent amplicon contamination. Photochemical inactivation also prevents aplicon contamination. At the present time, it can be said for all PCR-based approaches for species identification that strict quality control of PCR reaction mixtures and monitoring of the performance of the amplification itself is essential if PCR is used for diagnostic purposes. Recently a commercial PCR amplification kit for detection and identification of *M. tuberculosis* has been developed (Amplicor, Hoffman-LA Roche, Basel, Switzerland). The target for the PCR is the 16s rRNA sequence. The detection system requires hybridization with a *M. tuberculosis* complex-specific capture probe in a microplate format.

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 of 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 are 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[15,16].

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[17].

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[18,19]. Recently,

resistance to rifampicin was found to be determined by mutations causing change in the rpo B gene coding for Beta-subunit of RNA polymerase[21]. Streptomycin-resistant mutants show alterations in the ribosomal protein S12 and/or in the highly conserved rspl gene, which encodes the S12 ribosomal protein. 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[22]. Quinolones and coumarins are the known inhibitors of the DNA gyrase. The gyrA mutations have been found to be associated with resistance to quinolones[23].

Techniques such as single strand cellular polymorphism (SSCP) could identify mutations is short pieces 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[24].

A new technique called the Line Probe Assay (LiPA) also has been evolved using PCR to amplify the region of RNA polymerase involved in rifampicin resistance. A set of oligonucleotide probes are immobilized onto a membrane strip and rifampicin resistance can be assessed from the pattern obtained following hybridization. This technique is undergoing rigorous analysis at the moment[25].

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 [26,27]. An evaluation of a new rapid bacteriophage-based method for the drug susceptibility testing of *M. tuberculosis* has been reported recently by Wilson S M *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.

Antibody detection

In this technique, antibodies against mycobacterial antigens in sera from patients by enzyme-linked immunosorbent assay (ELISA) is done using polyclonal or monoclonal antibodies. When crude mycobacterial preparation are used for these tests, the test has low specificity. False-positive results are likely to be due to cross-reactions by environmental mycobacteria and other bacteria. To prepare purified antigens from mycobacterial cultures, techniques such as ion-exchange chromatography, gel filtration, isoelectric focusing, electrophoresis and affinity chromatography 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* [28].

At this point it is worthwhile to mention that the immune response is mycobacterial disease appears to be associated with HLA class type II allotypes and different patients appear to recognize different antigens[29]. This suggests that it is unlikely that all tuberculosis patients will recognize a single antigen. This may prove to be a handicap as far as antibody-based detection systems for mycobacteria are concerned[30].

DNA strain typing using restricted fragment length polymorphism (RFLP)

The principle behind the RFLP technique is that if a single base difference between otherwise two identical pieces of double stranded DNA is lying within the recognition site of restriction endonuclease, then digestion of both the samples with that restriction endonuclease will produce different products which can be resolved by electrophoresis resulting in different handling patterns called as Genomic or DNA fingerprints. Differences in banding patterns are referred to as RFLPs. RFLP typing of *M. tuberculosis* isolates is useful for epidemiological investigations in the spread of particular strains, especially multi-drug resistant strains and also to learn about relapses following successful treatment whether it is due to endogenous reactivation or exogenous infection[31,32].

Conclusions

The epidemiological situation of tuberculosis in India is complex with a very wide variation in the annual risk of infection, prevalence of disease and the pattern of antituberculosis drug resistance. Besides, the concentration of the disease among young age group makes tuberculosis as a major socio-economic problem. The burden is likely to increase with the onslaught of HIV epidemic with an increase of cases having dual infection, increase in morbidity and mortality due to tuberculosis. Improvement in case

detection will require development of rapid diagnostic tools with the aim to increase sensitivity, specificity and technical simplicity. This is vital in cutting the chain of transmission among the vulnerable population.

Today, many new techniques are available for the diagnosis of tuberculosis and also for detection and identification of *M. tuberculosis.* However, detection of AFB by direct microscopy and identification of cultured mycobacteria by biochemical methods still remain the recommended methods. Faster culture methods using radiometric systems such as Bactec etc. 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 chance 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.

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