

Diagnosis of MDR Tuberculosis

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Abstract: Tuberculosis is responsible for 7% of all adult deaths and 25% of preventable adult deaths. Multi-drug resistant tuberculosis strains are viewed as mainly a problem of the developing world and are resistant to at least two drugs, INH and Rifampicin. Acquired resistance or secondary resistance is more common than primary resistance. Primary drug resistance is that when a patient who has not been previously on anti tubercular therapy (ATT) and develops resistance whereas acquired resistance develops while on an inappropriate ATT regimen. The frequency of multi drug resistance varies geographically. High resistance was found in erstwhile countries of the USSR, the Baltic Republics, Argentina, Nepal and China. In our centre resistance observed has been 5%-7%. Survey of initial MDR TB cases in community is important for redesigning the effective regimen for new cases and also for planning appropriate regimen for cases requiring re-treatment.

Introduction

According to WHO, approximately 7 to 8 million people have tuberculosis and around 3 million die annually due to this infection. In India about 2 million cases are infected every year. Multi-drug resistant tuberculosis (MDR-TB) strains are resistant to at least two drugs i.e. INH and rifampicin; frequency of such resistance varies geographically (Table 1). MDR-TB is being highlighted as a problem of the developing world; prevalence varies from 14.5% in Korea to 33.8% Gujarat (India); In our centre drug resistance varies from 5 to 7 percent (Fig.1).

Table 1 : Percent Drug resistance from different parts of World and India².

Place/Year of Reporting	One or more drug	INH	Rifampicin	Strepto mycin	EMB	PZA	MDR	Remarks
Japan 2003	1.9	0.81	5.1		0.81		0.32	Primary
	9.7	11.5	7.3		2.4		6.1	Acquired
Madagascar (2000)							5	Acquired
Gambia 2003	4							
China 2002	17.5						2.1	Primary
Ajajbeijan Prison (2001)							52.3	-
Central India 2001 (4)		54.2		41.5		50	8.1	-
Saudi Arabia 2002	50	9.1	2.8	3.6	1.6	5	2.8	-
Jodhpur 2000 (5)		16.67	6.67	16.67	6.67		3.3	Primary
		61.76	70.59	51.52	39.39		38.2	Acquired

EMB: Ethambutol, PZA: Pyrazinamide; MDR: Multidrug resistance

It is essential to understand that for **laboratory diagnosis** of MDR-TB, the most important step is the firm diagnosis of tuberculosis; the question of its resistance needs be settled later. This is also a fact that in certain percent cases culture is not positive for *M. tuberculosis*. Since it is not easy to achieve the gold standard there is a consensus evolving round the world whether culture - so far gold standard, needs to be altered in favour of compelling clinical evidence fortified with newer molecular tools.

To diagnose MDR-TB nothing different needs to be done than the methods used for diagnosing a sensitive strain of *M. tuberculosis*. But identification of acid-fast bacilli (AFB) with regard to its species is equally important. The role of NIM has become more pronounced with the advent of the scourge of HIV, more so in

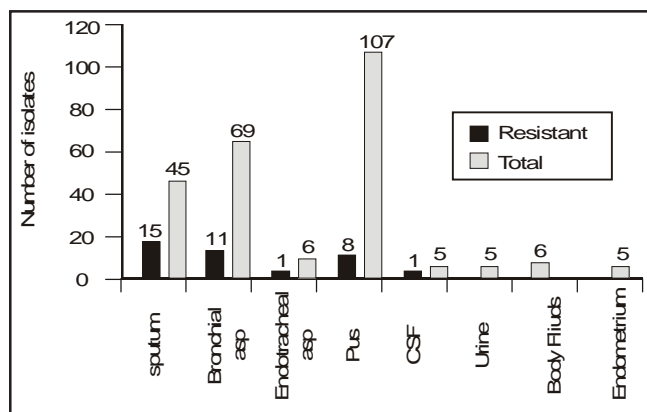


Fig. 1 : Resistant isolates at SGRH

advanced countries where tuberculosis had been brought under control. However, in developing countries where tuberculosis is still rampant, atypical mycobacteria are still a minority. There is no evidence in their being transmitted directly from man to man. They are generally resistant to the antitubercular drugs and might be diagnosed as MDR-TB. As the generation time of mycobacteria is much longer than the other usual pathogenic organisms, advent of rapid culture methods along with molecular techniques can produce faster results with speculation as well. Table 1 gives the prevalence of drug resistance (MDR) in India and abroad.

Sample Collection

Sputum is the most frequently examined specimen for the detection of AFB, it is important to evaluate sufficient number of samples from each patient to ensure recovery of even low numbers of mycobacteria. Besides this, the quality of the specimen is of paramount importance. Clinician should spend time explaining to the patient the method of producing good quality sputum. Three deeply expectorated morning sputum samples on three consecutive days are usually adequate.

When sputum is unavailable or the findings are constantly negative in patients with convincing clinical evidence of pulmonary tuberculosis, gastric lavage may be necessary and such samples must reach the laboratory promptly since mycobacteria do not survive for long period in acidic gastric washing. The recommended guidelines for the collection of various types of samples are given in Table 2. The significance of representative sample collection in adequate quantity and with minimum

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transportation time helps in proper evaluation for mycobacterial disease. This is more true for samples like C.S.F., pleural fluid and other body fluids. (Table 2)

Table 2 : Recommended guidelines for sample collection.

Aspirates-Fluids	Sterile syringe, container, or direct inoculation on culture media.
Blood, Bonemarrow	Isolator or direct inoculation on culture media
BAL	Sterile cotainer
Gastric lavage	Sterile container with 100mg of sodium carbonate; may be useful if sputum is difficult to obtain.
Sputum	Expectorate from lungs by a productive cough (not saliva), sterile container.
Stool	Clean container
Tissues biopsy specimens	Sterile container, do not add formalin
Urine	Firt whole morning void at least 100ml; do not pool over 24 hours.

(Reproduced from suppl. Indian J Paediatr 2002;69:S11-S19)

Microscopy

It is a rapid and the best method to identify the presence of the organism viable or nonviable in a clinical sample. It has been estimated that when using concentration techniques approximately 10^4 AFB/ml of sputum are required for a microscopy to be positive. Patients with extensive disease shed large number of mycobacteria with good correlation between positive culture and smear. In patients with minimal or less advanced disease the correlation of positive smears to positive cultures may be only 25%-40%¹.

Two types of acid fast staining can be used: (i) *Ziehl-Neelson (ZN)* and (ii) *Auramine-Rhodamine (AR) Fluorochrome staining*. Approximately 18% of ZN smear negative but culture positive patients can in addition be diagnosed using A-R staining². Similar results were also seen in SGRH as reflected in fig 2. Dead mycobacterial cells will also stain with fluorochrome stain leading to smear positive and culture negative situation in approx. 10% of cases and need be kept in mind while assessing treatment efficacy. Smear positive does not indicate treatment failure².

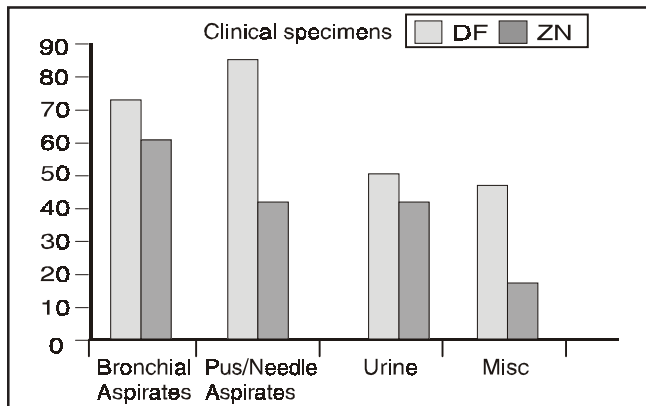


Fig. 2 : Comparative analysis of DF and ZN in SGRH

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Culture

1 Conventional Mycobacterial Culture : As low as 10-100 bacilli/ml can result in a culture positivity. It is mandatory that all highly suspect samples be subjected to culture. A variety of

methods and media are in use to culture mycobacteria in-vitro. The most popular media in clinical laboratory is Lowenstein Jensen as a solid media and Middlebrook 7H9 being its liquid counterpart. this age-old method of mycobacterial culture takes a very long time i.e. 6 to 8 weeks (average 21 to 28 days) for *Mycobacterium tuberculosis* to form colonies & the same time is required for sensitivity in this medium. Thus a total of 12-16 weeks is required for culture and sensitivity using this method.

2 Rapid Mycobacterial culture methods :

2a Bactec Systems/MB-BacT : Substantial improvement in the time to detection and the total number of positive cultures can be achieved if automated or semi-automated liquid culture systems such as BACTEC™ 460TB that use Middlebrook 7H12 is used. This radiometric system can detect growth much earlier than the eye can see on solid conventional media (LJ). Widely used BACTEC™ 460TB system utilises 7H12 broth containing Carbon 14(¹⁴C) labeled palmitic acid along with the mixture of antibiotics (PANIA, BBL). The growth can be ascertained by the liberation of ¹⁴C₂ by the mycobacteria. But other contaminant bacteria can also break down palmitic acid and result in false positive results.

New systems that rely on non-radiometric growth have been developed such as MB BacT/3D (bioMerieux) (fig.3), Mycobacterial Growth Indicator Tube (MGIT-Becton Dickinson) and BACTEC™ 9000TB automated culture systems, appear to be more promising. The time required for isolation is reduced from 6 weeks to 3 weeks. In addition the turn around time using BACTEC/MB BacT-3D systems is reduced; smear positive specimens can be positive within 8 days as compared to 18 days in conventional medium In smear negative samples the mean time to detection with rapid culture is 14 to 16 days as compared to 26 days for the conventional medium³. Antibiotic sensitivity also can be done using these instruments by adding a known MIC of the drug to

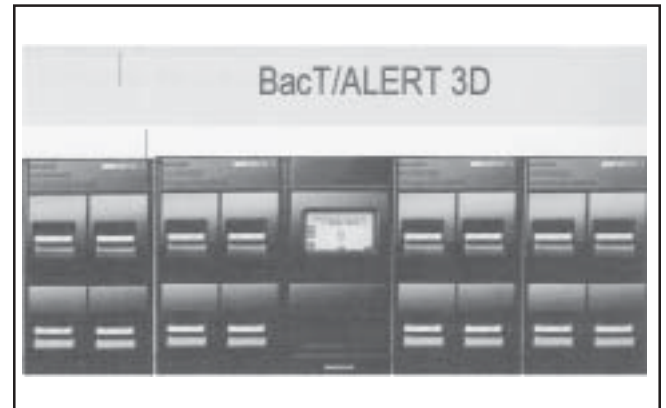


Fig. 3

the liquid media & reduce mean time for detection o almost 11 to 18 day³.

2b Septi-Check System : Another broth based system introduced is Septi-Chek AFB (BBL) which uses a biphasic media and provides rapid growth and drug susceptibility without need for routine subculturing. Detection time is shorter than with conventional agar but significantly longer than the BACTEC/MB-BacT-3D systems.

2c Isolator lysis centrifugation system : The isolator tube (WAMPOLE) provides a unique approach to the recovery of mycobacteria from blood. Approximately 10ml of blood is collected into the Isolator tube containing an anticoagulant and saponin,

which will lyse the red blood cells, releasing any microbe within. After centrifugation at 3000x g for 30 minutes, the sediment is removed and inoculated into a variety of automated mycobacterial system and 80% isolats were recovered with the mean time to detection as 14.4 days.

2d The Luciferase Reporter mycobacteriophage (LRP) test - a virus that infects mycobacterium has a cloned gene responsible for production of Luciferase Reporter enzyme which emits light in the presence of mycobacterium. This method is very sensitive, specific and rapid test and is equally effective as MGIT 960 in detecting MTB⁵.

Identification

a Conventional methods for identification rely on morphology and biochemical reactions like tube catalase at 68°C, niacin, and nitrate tests which are cumbersome and could take as long as two months using standard methods, in contrast to accuprobe which identifies it from culture in less than an hour.

b Rapid Mycobacterial identification techniques

bl. the Accuprobe (GenProbe, USA) system (fig.4) : Tests for the identification of MIB complex is a rapid DNA probe test which utilizes the technique of nucleic acid hybridization for the specific identification of MIB complex isolated from the culture. The TB complex includes following species; *M.tuberculosis*, *M.bovis*, *M.bovis BCG*, *M.africanum*, *M.microti*, and *M.canettii*⁶. *M.tuberculosis* is most common pathogen isolated from humans, *M.bovis BCG* may be transmitted from infected animal to humans, and the others primarily infects animals. *M.africanum* causes pulmonary tuberculosis is tropical Africa. For most clinical laboratories identification of an isolate as TB Complex is sufficient because the probability that an isolate is a species other than *M.tuberculosis* is extremely small⁷.



Fig. 4 : Accuprobe Luminometer (GEN-PROBE)

Principle - The accuprobe system uses a single stranded DNA probe with a chemiluminescent label that is complementary to the ribosomal RNA of the target organism. After the RNA is released from the organism it forms a stable RNA-DNA hybrid. The selection reagent allows for the differentiation of non hybridised and hybridized probe. The labeled DNA-RNA hybrid are measured in a luminometer. A positive result is aluminometer reading equal to or greater than the cut off. A value below the cut off is a negative result.

In Our experience at SGH : Out of 38 smear positive samples processed since June 15 - Dec 9, 2004, 29 were Bactalert 3D (bio-Merieux, France) culture positive and subsequently accuprobe

positive for MIB - complex; whereas 5 culture positive were accuprobe negative for MIB; thereby indicating that 5 smears and culture positive cases were NIM, if not speciated would have been presumed to be *M.tuberculosis* and treated with no or diminished response to ATT. Such cases run a risk of being labelled as MDR-TB. Break up details of NIM positive cases can be obtained from figure 5.

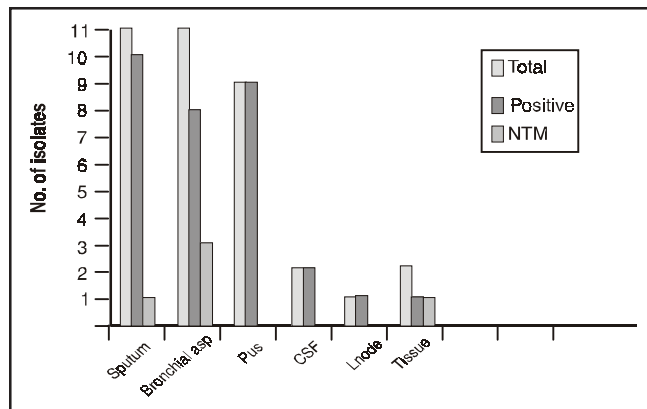


Fig. 5 : Accuprobe results

Molecular Assays in the Diagnosis of Tuberculosis

Amplification assays - DNA or RNA based.

A. DNA amplification assays

1 Polymerase chain reaction (PCR) : First described in 1988, it was used to detect *M.tuberculosis* from the clinical samples like C.S.F., urine and other body fluid and blood using molecular methods. A positive PCR result using *M. tuberculosis* specific primers on a smear positive sample would indicate that AFB seen were *M.tuberculosis* whereas a negative PCR would suggest an NIM, as long as proper controls are included in the assays. With a smear negative specimen, however a negative PCR does not exclude the presence of *M.tuberculosis*. Inhibitors are known to exist in CSF or pus samples that can inhibit amplification in any molecular assay⁸. PCR cannot differentiate between the dead or live mycobacteria.

2 Other DNA application assays : are Ligase Chain reaction LCR, Strand displacement assay SDA and ϕ replicase amplification which has not gained as much prominence as PCR. Advantage of these assays are that they are sensitive but cannot differentiate dead from a living bacilli.

B. RNA amplification assays -

1 NASBA : Nucleic acid sequence based amplification assay (NASBA) used in identifying MIB and NIM (from a positive bottle) using specific probes. (NASBA; bio-Merieux/Organon & Technika).

2 TMA: Transcription mediated amplification (TMA; Genprobe, bio-Merieux).

Both are isothermal amplification methods. Instead of using DNA as the target, uses 16S rRNA, which is more abundant than the corresponding DNA making it more sensitive than PCR. The rRNA is transcribed by a reverse transcriptase forming a transcription complex from which isothermal amplification of the target sequence is catalysed by RNA polymerase. The amplified nucleic acid is detected using electro-chemiluminescence technique. The sensitivity and specificity of such tests have been well established in both single and comparative evaluations. The sensitivity and specificity for most of the above tests fall within

the same range of values given for PCR.

Advantage over DNA assays : The detection of *Mycobacterium tuberculosis* By NASBA 16S rRNA also gives an added information regarding the viability of the organism as compared to the DNA based PCR technique which could pick up even dead sequences as mentioned earlier.

C Other Assay available commercially as a kit is (LiPA MYCOBACTERIA; Innogenetics Zwijndrecht, Belgium) based on reverse hybridisation system principle⁹. PCR is used to amplify the 16S-23S ribosomal spacer region and the product is hybridised to probes bound to a nylon strip; binding is identified colorimetrically. Unlike acuprobe, a single test can identify the range of species using specific probes. This technique can be used for identification of mycobacteria isolated from MB BacT/3D system.

Needless to say, appropriate infrastructure, staff training and quality control procedures are essential to avoid cross contamination events leading to false positive results in all the above mentioned molecular assays. Moreover the laboratory report is as good as the quality of the specimen submitted.

Seroassay for MIB

ELISA for TB was found positive in 79.4% of sputum AFB positive patients but was also positive in 56.2% of AFB negative sputum samples¹⁰. All seroassays must be evaluated in light of the clinical evidence and should not be taken as the sole evidence of infection. Seroassay moreover has no role in diagnosing MDR-TB.

Detection of Drug Resistance

There is a limitation in adequate interpretation of drug resistance due to lack of standardisation of laboratory procedures resulting in misleading reports with poor reproducibility, as a result a poor knowledge of the true incidence of drug resistance exist.

There are three widely used methods for drug sensitivity on the Lowenstein-Jensen medium:

1 The absolute concentration method also termed as minimum inhibitory concentration (MIC) method - a standardised inoculum of the organism is inoculated on a drug free media (control) and media containing graded concentration of the drug as a test. Resistance is expressed as the lowest concentration of the drug that inhibits growth on all or almost all tubes indicating MIC.

2 The resistance ratio method : Resistance of the test organism is compared with that of a standard laboratory strain and expressed as the ratio of the MIC of the test strain with that of the standard strain against each drug.

3 The proportion method : If the number of colonies at the known MIC of a drug is > 1% of the colonies grown in drug free medium then the strain is considered clinically resistant. Culture and sensitivity test can also be done using other medias like 7H9, 7H10 or 7H11 Middlebrook. A direct sensitivity on smear positive cases where the drug can be incorporated in the medium can hasten the results to 6-8 weeks, which otherwise could take 12-16 weeks.

4 E-Test : It is a gradient minimum inhibitory concentration technique and is found to be quick, accurate, reliable and easy to perform and the results are available between 7-10 days. Agreement between L-J and E test methods is 87% while between L-J and MH11 methods is 79%¹¹. It is especially of great value in drug resistant cases where MIC is important. Hausdorfer et al demonstrated more than 90% correlation between E test and agar proportion method for all the four first line antitubercular drugs¹².

Automated methods currently used for rapid susceptibility testing

of *M. tuberculosis* include MB-Bactalert-3D, BACTECTM 460 radiometric method etc.

Molecular Diagnosis of Antimicrobial Resistance

The frequency of resistance mutations has been estimated to be 1 in 10⁸ for R & 1 in 10⁶ for H, respectively and 1 in 10⁵ for S, EMB, para aminosalicylic acid (PAS), ethinamide (N), cycloserine and thiazetazone.

Rifampicin : More than 95% of rifampicin-resistant MIB isolates have mutations in rpoB, the gene encoding the RNA polymerase β -subunit¹³. INH-Resistance to INH is more complex. Many resistant organisms have mutations in the katG gene encoding catalase-peroxidase that result in altered enzyme structure¹⁴. S resistant strains have a mutation on the rrs and rpsL gene encoding a 16S rRNA and a S12 ribosomal subunit protein, respectively¹⁵. Resistance to pyrazinamide (Z) is caused by mutations in the gene pncA, encoding pyrazinamidase resulting in diminished enzyme activity.

Ethambutol resistance in approximately 60% of organisms is due to amino acid replacements at position 306 of an arabinosyl transferase encoded by the embB gene.

Similarly **Fluoroquinolones** resistance is associated by gyrA gene mutation. Kanamycin resistance is due to nucleotide substitutions in the rrs encoding 16S rRNA.

Various molecular probes for detection of such resistance genes are being used for predicting resistance but are still not available for commercial use.

The Future

The current knowledge does not permit utilisation of molecular resistance detection assays as a matter of routine. Molecular assays have a good correlation in smear positive cases only and is recommended for use only in such cases at present by FDA (USA). The issue of quality control (QC) in molecular assays remains yet to be resolved due to the absence of the availability of universally acceptable standard controls allowing acceptable reproducibility of the assay world over.

However genotypic analysis involving amplifications of the genomic region conferring resistance-using PCR, followed by post amplification analysis of mutation causing drug resistance by single strand conformation polymorphism (PCR-SSCP) can reduce the turn around time. Reverse hybridisation based line probe assay also have been used reliably to detect the mutations causing drug resistance. Molecular beacon assays is used in real time PCR to detect as little as a single nucleotide substitution causing drug resistance can be used¹⁶. The only silver lining in the name of molecular diagnosis of MDR-TB could be the detection of rifampicin or INH resistant cases earlier. early case detection could help in containment of the spread of MDR-TB strains.

Other methods, which can also be used to detect drug resistance, are heteroduplex analysis, dideoxyfingerprinting, an RNA/RNA duplex base pair mismatch assay, rRNA/DNA bioluminescence labeled probe assay and luciferase mycobacteriophage assay in which only viable mycobacteria can allow replication of the phage whereas the dead ones cannot. Isolate grown in the presence of drug will not emit light on addition of luciferin substrate if it is susceptible whereas resistant isolate will.

A commercial kit for detection of mutated gene for resistance would serve as a valid surrogate marker for MDR-TB.

Based on diverse lines of evidence, the fitness estimates of drug-resistant *M. tuberculosis* are quite heterogeneous and may preclude

the ability to predict future trends of this pathogen¹⁷.

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