

WHAT IS NEW IN THE DIAGNOSIS OF TUBERCULOSIS?*

PART II; TECHNIQUES FOR DRUG SUSCEPTIBILITY TESTING

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Drug susceptibility testing can be performed based on mycobacterial cultivation on solid media, either egg or agar-based. In the direct test a set of drug-containing and drug-free media is inoculated directly with concentrated specimen. The advantage of the direct method over indirect method is that the results are available sooner (within 3 weeks on agar plates), and better represent the patient's original bacterial population

Indirect Method

In the indirect test, the pure culture is inoculated in drug containing and drug-free slopes either in egg-based Lowenstein-Jensen medium or agar based 7H11 medium.

PHENOTYPIC METHODS*Absolute concentration method*

This method uses a standardized inoculum grown on drug free media and media containing graded concentrations of the drug(s) to be tested. Several concentrations of each drug are tested, and resistance is expressed in terms of the lowest concentration of the drug that inhibits growth; i.e. minimal inhibitory concentration (MIC). This method is greatly affected by the viability of the organism.

Resistance ratio method

This would compares the growth of

unknown strains of tubercle bacilli with that of standard laboratory strain (H37Rv). Parallel sets of media containing two-fold dilutions of the drug are inoculated with standard strains of tubercle bacilli. Resistance is expressed as the ratio of the MIC of the test strain to the MIC of the standard strain in the same set. This test is also greatly affected by the inoculum size as well as the viability of the strains. In addition, any variation in the susceptibility of the standard strain also affects the RR of the test strain.

Proportion method

This method enables a precise estimation of the proportion of mutants resistant to a given drug. Several 10-fold dilutions of inoculum are planted on to both control (drug-free) and drug-containing media; at least one dilution should yield isolated countable (50-100) colonies. When these numbers are adjusted by multiplying by the dilution of the inoculum used, the total number of viable colonies on the control medium, and the number of mutant colonies resistant to the drug concentrations tested may be estimated. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant portion as a percentage of the total population used. The proportion method is currently the method of choice for estimating drug resistance and this principle is being applied to the following rapid testing methods:

- (i) BACTEC 460 (First and second line);
- (ii) MGIT 960;
- (iii) MB/BacT system; and
- (iv) ESP II system

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RECENTLY DEVELOPED PHENOTYPIC METHODS

E-test (Commercially available as AB BIODISK)

The E-test is based on determination of drug susceptibility using strips containing gradients of impregnated antibiotics. There are reports about a high rate of false resistance by this method when compared with BACTEC or conventional LJ proportion methods¹.

Microwell Alamar blue assay and microplate tetrazolium reduction assay

These tests are colorimetric based on the oxidation-reduction of the dye Alamar blue or MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Drug resistance is detected by the reduction of the dye from blue to pink due to the oxidation-reduction metabolism of viable organisms^{2,3}.

Mycolic acid index susceptibility testing

This is a modification of the original mycolic acid analysis by HPLC where a coumarin compound is used as a fluorescent derivatizing agent of mycolic acid instead of p-bromophenacyl bromide. The drug sensitivity is assessed by measuring the total area under mycolic acid (TAMA) chromatographic peaks of a culture of *M.tuberculosis*, and this area has a very good correlation with log CFU per milliliter. Depending on the signal and quantification of this procedure, drug susceptibility pattern can be carried out as a rapid method⁴.

Microscopic observation of broth cultures - Drug susceptibility assay

This novel method of microscopic observation of broth culture with drugs is used for drug sensitivity testing. It is a relatively inexpensive and fairly rapid drug susceptibility testing method with a high sensitivity and specificity and is suited for disease endemic developing countries⁵.

Micro-colony detection

This employs observation of micro-colonies

of *M.tuberculosis* with the help of a microscope, on a thin layer of 7H11 agar plate and is used for drug sensitivity testing. It is less expensive than the conventional proportion method, and may be a good low-cost alternative for resource poor countries⁶.

Pha B assay

This is a new phenotypic culture drug susceptibility testing method named as phage amplified biologically (pha B), and is based on the ability of viable *M.tuberculosis* to support the replication of an infecting mycobacteriophage; non-infecting exogenous phages are inactivated by chemical treatment. The number of endogenous phages, which is an indication of the original number of viable *M.tuberculosis*, is determined after cycles of infection, replication and release in rapidly growing mycobacteria. In the case of drug-resistant *M.tuberculosis*, bacilli will remain viable and protect the mycobacteriophage. Any mycobacteriophage protected within viable bacilli replicate and ultimately lyse their host. For rapid detection, the released mycobacteriophages are mixed with 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 culture of *M.smegmatis*. The number of plaques generated from a given sample is directly proportional to the number of protected mycobacteriophages, which is dependent on the number of tubercle bacilli that remain viable after drug treatment (i.e. drug-resistant). Gingeras et al⁷ reported successful application of this assay.

Luciferase reporter phage assay

In this technique, viable mycobacteria are infected with reporter phages expressing firefly luciferase gene. Easily detectable signals are seen a few minutes after the 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 phages⁸. The other reporter molecule described is the green fluorescence protein (GFP) of the jellyfish *Aequorea Victoria*. This reporter system does not require co-factors or substances due to intrinsic fluorescence nature of the GFP⁹. These tests have generally good sensitivity and reproducibility but are yet to be used in routine clinical laboratories.

GENOTYPIC METHODS

These are essentially required for the rapid identification of multi drug resistant (MDR) TB strains. In contrast to other bacteria, drug resistance in *M.tuberculosis* is not plasmid mediated. Most of the molecular events relating to the chromosomal basis of drug resistance have been elucidated. The

phrase “MDR state” in mycobacteriology refers to simultaneous resistance to at least Rifampicin and INH (with or without resistance to other drugs). Genetic and molecular analysis of drug resistance in MDR TB suggests that the bacilli usually acquire resistance either by alteration of the drug target by mutation or by titration of the drug through over-production of the target. MDR TB usually results from accumulation of individual target genes¹⁰. The list of various gene loci conferring resistance in *M.tuberculosis* are listed in the accompanying table.

Important genotypic drug susceptibility testing methods are given as under:

Automated DNA sequencing

Among the molecular techniques available

Table: Various gene loci conferring drug resistance in *M.tuberculosis*

Drug	Gene	Gene product/functional role	Cellular target
Rifampicin	rpoB	B sub-unit of RNA polymerase/transcription	Nucleic acids
Isoniazid	KatG	Catalase-peroxidase/Activation of pro-drug	Cell wall
	OxyR-ahpC Kas A	Alkyl-hydro-reductase β -ketoacyl acyl carrier protein	
INH-Ethionamide	inhA	Enoyl-ACP reductase/Synthase; Mycolic acid Biosynthesis	Cell wall
Streptomycin	rpsL	Ribosomal protein S12/Translation	Protein Synthesis
	rrs	16s rRNA/translation	
Fluroquinolone	gyrA	DNA gyrase	Nucleic acids
Pyrazinamide	pncA	Amidase/Activation of pro-drug	Unknown
Ethambutol	embCAB	Arabinosyl transferase/Arabinan; polymerization	Cell wall

to detect *M. tuberculosis* drug resistance, DNA sequencing of PCR amplified products are most widely used and is becoming the gold standard for this purpose. It has been performed by both manual and automated procedures, though the latter is most commonly used. The DNA sequencing is used for characterization of the mutation responsible for drug resistance. This technique is being mainly used for drugs like Rifampicin, Isoniazid, Streptomycin and Ciprofloxacin¹¹.

PCR SSCP

PCR SSCP is based on the property of single-stranded DNA to fold into a tertiary structure whose shape depends on its sequence. Single strands of DNA differing by only one or a few bases will fold into different conformations with different mobilities on a gel, producing what is called a single strand conformation polymorphism (SSCP). In combination with PCR, SSCP has been applied for the detection of resistance to Rifampicin, Isoniazid, Streptomycin and Ciprofloxacin¹². The whole process can be automated for use in large reference laboratories.

PCR HDF

This assay is performed by mixing amplified DNA from the test organisms and susceptible control strains to obtain hybrid complementary DNA. If a resistant strain is present, the mutation will produce a heteroduplex which has different electrophoretic mobility compared with the homoduplex hybrid (no mutation present). The PCR-HDF is used to detect all Rifampicin resistant strains having mutation within the 305-bp region of the *rpo B* gene¹³. This assay can be used cost-effectively only in reference laboratories with a large number of specimens where the cost of the test per specimen can be reduced.

LiPA (Solid phase hybridization assay)

The line probe assay or LiPA is a commercial test for the rapid detection of *M. tuberculosis* complex and Rifampicin resistance. The LiPA is based on the hybridization of amplified DNA from the cultured strains or clinical specimens to ten probes

encompassing the core region of the *rpo B* gene of *M. tuberculosis*, which is immobilized on a nitrocellulose strip. The absence of hybridization of the amplified DNA to any of the sensitive sequence-specific probes indicates mutations that may encode resistance; likewise, if hybridization to the mutation-specific probes occurs, the mutation is present¹⁴.

Miscellaneous genotypic methods

These include new genotypic techniques for the rapid detection of drug resistance in *M. tuberculosis*. Cleavage fragment length polymorphism (CFLP)¹⁵, dideoxy fingerprinting (ddf)¹⁶, hybridization protection assays¹⁷, a technique based on reverse transcriptase-strand displacement amplification of m-RNA¹⁸, RNA-RNA duplex base-pair mismatch assay¹⁹ and DNA sequence analysis using fluorogenic reporter molecules²⁰ (i.e. molecular beacons). However, these techniques have not been extensively studied and have not been further validated with clinical isolates. Although they share a high specificity common to all sequencing techniques, most of them rely on technically demanding procedures and in some cases need specialized and costly equipment precluding their use at the clinical laboratory level, not to mention mycobacteriology laboratories in developing countries where TB is more prevalent.

DNA strain typing using 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 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 relapse following successful treatment, and to know whether it is

due to endogenous reactivation or exogenous reinfection²¹.

CONCLUSION

Many new possibilities have emerged for the detection of drug resistance in *M.tuberculosis* and for performing drug susceptibility tests. These novel methods rely on new information concerning molecular mechanisms of drug resistance, or on new approaches in detecting mycobacterial growth. The genotypic methods have the advantage of being rapid and specific. However, not all of the molecular mechanisms of drug resistance are known; hence the current molecular tools cannot detect all resistant strains. The phenotypic methods are more diverse; some of them though simple in their procedure, still require expensive equipment, not always available in laboratories of TB endemic countries. Some of them are very useful, being low-tech and simple so that they can be routinely used in developing countries, while others need further evaluation and validation to obtain acceptable levels of sensitivity, specificity and reproducibility before they replace the current drug susceptibility test procedures.

Today, although many new techniques are available for the diagnosis of TB and also for detection and identification of *M.tuberculosis*, detection of AFB by direct microscopy is the only feasible method recommended for the tuberculosis control programme of India in detecting infectious pulmonary tuberculosis cases and for monitoring the progress of patients during treatment. Wherever facilities are available, isolation of mycobacteria by culture, and drug sensitivity testing by conventional methods to detect MDR TB cases still remain the recommended methods in disease endemic countries. Faster culture methods using radiometric systems such as BACTEC or non-radiometric systems like MGIT, etc. are being used increasingly mainly because they reduce the time needed for culture and drug sensitivity testing to about 2-3 weeks. Nucleic acid amplification techniques are used 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 noted that most of the new techniques described involve prohibitive expenditure in terms of instrumentation, expertise and reagents, putting them out of reach of many laboratories in developing countries including India.

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