

Molecular Techniques in the Diagnosis of Drug-resistant Tuberculosis

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Abstract

In the last few years, there has been considerable progress in our understanding of the mechanisms of action and resistance to anti-tuberculosis agents. To date, there is information about 11 genes involved in resistance to all major anti-tuberculous drugs in Mycobacterium tuberculosis. Mutations in katG, inhA and ahpC genes are found in up to 90% of isoniazid-resistant strains, rifampin resistance is associated (> 96%) with rpoB mutations, pyrazinamide resistance with pncA mutations (72% to 97%), ethambutol resistance with mutations in embB (47% to 65%), streptomycin resistance with rrs or rpsL mutations (70%), and fluoroquinolone resistance with gyrA substitutions (75% to 94%). Additional genes and mechanisms may play a role, particularly in association with lower levels of resistance. Based on this growing set of information, genotypic analysis of resistance is becoming a real possibility, and novel tests are being developed. Issues such as effectiveness, cost-efficiency, and appropriate setting for the implementation of these techniques are not yet established.

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Introduction

The emergence of multidrug-resistant tuberculosis (MDR-TB), generally defined as resistance to at least isoniazid (INH) and rifampin (RMP), has generated concern for the future of tuberculosis control.¹ The global magnitude of the problem is not well known. Most of the available studies are non-representative surveys of a population or a country, frequently failing to discriminate between primary and acquired resistance. In addition, some studies use sub-optimally standardised laboratory methodologies. However, available data. (Fig. 1) suggest that while multidrug-resistance may not be a widespread problem, it remains a public health threat in areas with high prevalence of tuberculosis and sub-optimal tuberculosis control programmes.¹ The highest rates of acquired MDR-TB have been reported from Nepal (48.0%), Gujarat, India (33.8%), New York City, USA (30.1%), Bolivia (15.3%), and Korea (14.5%).¹ The emergence of drug resistance has been associated with several factors, in particular, inadequate chemotherapy and the AIDS epidemic. The selective pressure of inappropriate chemotherapy results from improper prescription of drugs, non-standard treatment regimens, poor patient compliance, and unreliable drug supply.

Mycobacterial drug susceptibility testing is performed with the purpose of providing useful information for the treatment of the individual patient, and for the planning of wider-scale, population treatment strategies. In addition,

the surveillance of drug resistance is a good surrogate measure of the strength of tuberculosis control programmes.² However, technical problems in standardisation of drug susceptibility testing remain.² These are mostly derived from difficulties in establishing an appropriate inoculum size, the stability of drug compounds in different culture media, and the reliability of results obtained when testing some of the drugs, in particular, ethambutol and pyrazinamide.

Since minimal or no growth on culture is needed for genotypic assessment of resistance markers, tests based on this information would theoretically offer a number of advantages over conventional culture susceptibility testing: (i) turn-around time in days rather than weeks or months, (ii) possibilities for automation, and (iii) biohazard reduction in the laboratory.

In the present report we summarise available data on molecular targets suitable for clinical diagnosis, together with information on novel mutation detection tools.

Mechanisms of Resistance and Drug Targets in *Mycobacterium tuberculosis*

A considerable amount of work has been devoted in the last few years to understanding mechanisms of resistance, and to identifying the genes involved. Today, key genes involved in resistance to all major drugs have been identified.⁴ Resistance to INH has been linked to mutation in one of three genes in ≥90% of clinical resist-

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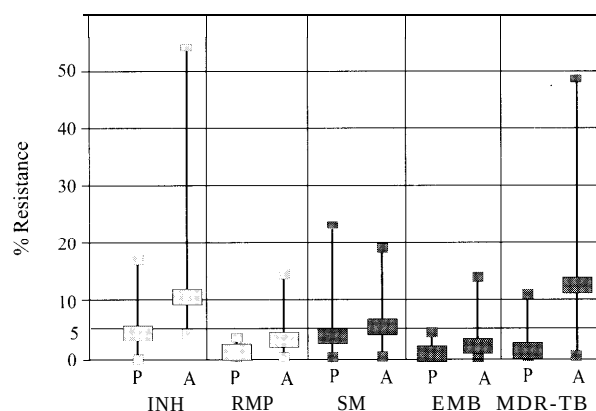


Fig. 1. Global rates of drug-resistance tuberculosis (WHO-IUATLD Global Surveillance Program 1985-1994). Represented are median, upper and lower range values reported in various studies. For each drug, primary and acquired resistance values are presented separately. INH: isoniazid; RMP: rifampin; SM: streptomycin; EMB: ethambutol; MDR-TB: multidrug-resistant tuberculosis.

ant isolates, resistance to RMP and pyrazinamide (PZA) to mutation in single genes, and high level resistance to ethambutol, fluoroquinolones and streptomycin to specific singular genes, while lower levels of resistance to these drugs may be attributed to other phenomena, such as efflux, permeability, or target over-expression.⁵⁻⁸ A summary of our current knowledge is presented in Table I.

Development of Novel Diagnostic Tools

Genotypic analysis involves amplification of the genomic region conferring resistance by the polymerase chain reaction (PCR), followed by postamplification analysis of mutations. Current methods, based on the variety of techniques described in Table II, require large amounts of amplification product in order to achieve unambiguous results. Although this limitation does not apply to analysis of cultured material, direct detection of drug resistance markers within clinical specimens may be limited by the presence of inhibitors within processed samples or by the small number of mycobacterial genomes within the starting material. Thus, positive Bactec cultures (growth index ≤ 100), liquid media, or colonies from solid media represent the optimal material for mutation analysis-direct testing on native material (e.g. sputum) is possible but restricted to strongly smear-positive samples.²⁴

Sample preparation should not represent an excessive workload. Several effective protocols can be applied that avoid labour-intensive DNA purification, but rely on simple bacterial lysis-boiling, mechanical disruption, or sonication.²⁴ Bio-hazard reduction prior to cell lysis is generally achieved by 20 minutes of incubation at 80°C; although, this step may not kill all organisms present.³³

TABLE I: MECHANISMS OF DRUG RESISTANCE IN *Mycobacterium tuberculosis*

Antimycobacterial agent	Mechanism of action	Genes involved in resistance	Frequency of mutations associated with resistance	Mechanism of resistance	Ref.
Isoniazid	Inhibition of mycolic acid biosynthesis	(i) <i>katG</i> (catalase- peroxidase)	(i) 42-58%	(i) Mutations in <i>katG</i> result in failure to generate an active intermediate of INH.	9-14
		(ii) <i>inhA</i> (enoyl-acyl carrier protein reductase)	(ii) 21-34%	(ii) Overexpression of <i>inhA</i> allows continuation of mycolic acid synthesis.	
		(iii) <i>ahpC</i> (alkyl hydroperoxide reductase)	(iii) 10-15%	(iii) <i>ahpC</i> mutations may just serve as a marker for lesions in <i>katG</i> .	
Rifampin	Inhibition of transcription	<i>rpoB</i> (β subunit of RNA polymerase)	96-98%	Mutations in <i>rpoB</i> prevent interaction with rifampin.	15
Streptomycin	Inhibition of protein synthesis	(i) <i>rpsL</i> (ribosomal protein S 12)	(i) 52-59%	Mutations prevent interaction with streptomycin. ^{5,16,17} Resistance not associated with mutation in <i>rpsL</i> or <i>rrs</i> is usually low-level.	
		(ii) <i>rrs</i> (16S rRNA)	(ii) 8-21%		
Ethambutol	Inhibition of arabinogalactan and lipoarabinomannan biosynthesis	<i>embCAB</i> (arabinosyl transferase)	47-65%	Overexpression or mutation of <i>embB</i> allow continuation of arabinan biosynthesis.	6,18,19
Pyrazinamide	Unknown	<i>pncA</i> (pyrazinamidase-nicotinamidase)	72-97%	Loss of pyrazinamidase activity results in decreased conversion of pyrazinamide to pyrazinoic acid, the putative active moiety.	20-22
Fluoroquinolones	Inhibition of the DNA gyrase	<i>gyrA</i> (DNA gyrase subunit A)	75-94%	Mutations in <i>gyrA</i> prevent interaction with fluoroquinolones. Mutations in <i>gyrB</i> , and efflux may contribute to resistance.	23

TABLE II: CHARACTERISTICS OF THE MAIN TECHNIQUES FOR DETECTION OF MUTATIONS^{4 24} ESTIMATES REFLECT APPROXIMATE MINIMUM COSTS AND TIME COMMITMENT, INCLUDING SAMPLE PREPARATION, PCR, AND MUTATION ANALYSIS. THERE IS LIMITED EXPERIENCE WITH OTHER REPORTED TECHNIQUES.^{25,26}

Technique	Steps	Basic cost per sample (US\$)	Hands-on/ Turn-around time (h)	Specific equipment needed	Comments	Ref.
Automated Sequencing	1) PCR of resistance region 2) Purification of PCR product 3) Sequencing reaction 4) Electrophoresis	10.00	8 / 48	Automated sequencer (approx. US\$100,000)	Gold standard. Cost effective alternative for limited number of samples if there is access to sequencing facilities.	27
PCR-SSCP	1) PCR of resistance region 2) Heat denaturation of PCR product 3) Electrophoresis	1.50 to 3.00	3.5 / 24	Can be performed "manually" using silver staining, or ethidium bromide (electrophoresis equipment approx. US\$5,000), or in an automated sequencer using fluorescence detection	Cheapest approach for evaluating numerous samples or multiple target genes simultaneously	28-30
Solid-phase Hybridization	1) PCR of resistance region 2) Hybridization to mutation-specific probes bound to nitrocellulose strip	NA	5.5 / <24	Shaking waterbath and strip microplate washer	Commercial kit* available for detection of rifampin resistance	12,31,32

PCR: polymerase chain reaction; SSCP: single-strand conformation polymorphism; NA: not available

* Innogenetics INNO-LiPA RTB

Most experience has been accumulated with sequencing analysis, the gold standard for mutation identification; and with two screening techniques: PCR-SSCP (single-strand conformation polymorphism) and a commercial kit for the detection of rifampin (Table II).

Other Rapid Techniques for Detection of Resistance

A novel approach to detecting drug resistance has recently been developed.³⁵ This biological assay is based on i) the infection of mycobacteria with a genetically altered phage carrying the firefly luciferase gene, and ii) the expression of the luciferase gene which degrades the substrate luciferin to produce light in viable cells. If the mycobacterium has been rendered non-viable or metabolically inactive by anti-tuberculous drugs, there will be no light emission. In contrast, drug-resistant strains will continue supporting the reaction, and emitting light. This approach has been suggested as a tool for screening for new anti-tuberculous drugs,^{35,36} but has not reached clinical application. More recently, another phage-based technology has been proposed as a possible alternative for the analysis of clinical material.³⁷

Solid-phase sequencing approaches, such as the use of the "DNA-chip", are promising techniques, though still in the developmental phase.^{38,39}

In Which Setting Should These Techniques be Implemented?

There are a number of limitations to the widespread use of PCR-based techniques. On one hand, resistance to anti-tuberculosis drugs generally involves changes at

various cellular targets. This would make testing for multiple agents difficult or impractical. On the other hand, the cost and technical demands of the equipment remain out of the reach of most laboratories.

There are, however, settings where the expedient identification of resistance may be highly desirable and prove to be cost-effective, in particular, in populations with a significant prevalence of MDR-TB and HIV co-infections, where data on the resistance genotype may result in better patient management and in a more effective utilisation of resources. For this purpose, the identification of mutations in the *rpoB* associated with rifampin resistance would serve as a valid surrogate marker for MDR-TB-resistance to this agent is generally associated with resistance to INH, and the presence of mutations can be efficiently evaluated given that they occur in a precise location in more than 96% of rifampin-resistant isolates. Although resistance to INH involves several genes, a limited targeting of selected regions-&G (codon 315), the regulatory regions of *inhA* and *ahpC*—allows detection of 90% of resistant isolates with three reactions.¹²

Regional or national TB control programmes could also benefit from the high output of some of these novel techniques, and from the fact that analysis is readily performed on heat-deactivated culture lysates, avoiding the need of shipping of bio-hazardous materials.

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