Multidrug-resistant Tuberculosis

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Chapter 9

Novel rapid antimicrobial susceptibility tests for *Mycobacterium tuberculosis*

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1. INTRODUCTION

Recent advances in the field of molecular biology and progress in the understanding of the molecular basis of *Mycobacterium tuberculosis* drug resistance have provided new tools for the rapid detection of drug resistance. However, partly due to the costs involved in their implementation, these novel techniques have not been applied in most clinical mycobacteriology laboratories, especially in low-income countries where tuberculosis (TB) constitutes a more serious health problem. Conventional methods such as the proportion method [1], the absolute concentration method, and the resistance-ratio method [2], are based on the measurement of growth in culture media containing antibiotics, and usually take several weeks to obtain results. The introduction of the BACTEC® radiometric system, and its adaptation for performing drug susceptibility testing (DST) of *M. tuberculosis* (BACTEC® TB-460), was therefore a major breakthrough for the rapid detection of mycobacterial growth and for the detection of drug resistance in tuberculosis [3,4]. BACTEC® is now used in numerous laboratories around the world but unfortunately mainly in developed countries or in reference laboratories with the necessary resources to implement this expensive mechanised technology as a routine procedure. With the current increase in drug resistance of *M. tuberculosis* [5], rapid and

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reliable DST methods are urgently required in the clinical mycobacteriology laboratory. Such techniques would not only aid patient management but also facilitate drug resistance surveillance, which is important in planning and evaluating TB control programmes.

TB diagnostics can be classified in two broad classes: genotypic methods and phenotypic methods. This chapter will review some techniques from both classes that have been developed recently for *M. tuberculosis* DST. Their applicability in different settings will also be discussed. The current conventional methods for DST are addressed in another chapter [6].

2. NOVEL GENOTYPIC TECHNIQUES

Insights into the molecular basis of drug resistance [7], combined with the availability of new molecular biology tools, have led to the development of several novel genotypic techniques for the rapid detection of *M. tuberculosis* drug resistance. Some of them make use of expensive equipment and laborious techniques, while others employ less sophistication, but all of them involve DNA extraction, gene amplification, and detection of a mutation. These techniques have several advantages: rapid turn-around time (TAT) of days instead of weeks, no need for growth of the organism, the possibility for direct application to clinical specimens, reduction in biohazard risks, and feasibility for automation. Unfortunately, the molecular tests also have disadvantages, including problems with inhibitors when attempting to apply these techniques directly to clinical samples. Following are some of the novel genotypic techniques described for detection of *M. tuberculosis* drug resistance.

2.1 Automated DNA sequencing

Among the molecular techniques available to detect *M. tuberculosis* drug resistance, DNA sequencing of PCR amplified products has been the most widely used, becoming a gold standard for this purpose. It has been performed by both manual and automated procedures although the latter has been the most commonly used [8-10]. Kapur et al. [11,12] described the application of automated DNA sequencing for characterizing mutations in the *rpo*B gene in rifampicin-resistant strains of *M. tuberculosis* and for the rapid identification of *M. tuberculosis* and *Mycobacterium* species. More recently Pai et al. [13] have confirmed the utility of the technique for *Mycobacterium* species assignment and for surrogate rifampicin susceptibility testing in a hospital-based clinical laboratory, where the rifampicin susceptibility phenotype was correctly predicted for all strains

evaluated. Automated DNA sequencing has also been used to detect mutations responsible for resistance to INH, ciprofloxacin and streptomycin [8,14,15].

2.2 PCR-single strand conformation polymorphism (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 single strand conformation polymorphisms (SSCP). After PCR amplification of the gene region of interest, the amplified DNA strands are separated by heat denaturation followed by cooling to allow single DNA strands to fold into a characteristic conformation, whose different electrophoresis mobility is detected on a gel.

In combination with PCR, SSCP has been applied for the detection of resistance to rifampicin, isoniazid, streptomycin and ciprofloxacin [8, 14-16]. More recently, Pretorius et al. [17] applied PCR-SSCP for rapid detection of rifampicin resistance in clinical isolates of *M. tuberculosis* and were able to detect 95% of the resistant isolates. Scarpellini et al. [18] also used PCR-SSCP to detect rifampicin resistance in cerebrospinal fluid samples, correctly identifying the rifampicin susceptibility phenotype of isolates from all patients for whom susceptibility tests were available.

As with automated sequencing, SSCP by automated methods seems more suitable for large reference laboratories in developed countries. An alternative approach using silver staining has also been proposed [9], which could probably be implemented in laboratories with limited resources.

2.3 PCR-heteroduplex formation (PCR-HDF)

This assay described by Williams et al. [19] is performed by mixing amplified DNA from 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 a different electrophoretic mobility compared with the homoduplex hybrid (no mutation present). PCR-HDF was employed to detect all rifampicin resistant isolates having mutations within a 305-bp region of the *rpo*B gene. The test does not require radioactive compounds and may be appropriate for clinical laboratories. More recently, Williams et al. [20] have applied a procedure utilizing a single tube hemi-nested PCR amplification generating a *M. tuberculosis*-specific *rpo*B fragment that is annealed to a synthetic universal heteroduplex

generator (UHG) derived from *rpo*B gene region where mutations encoding rifampicin resistance occur. From 44 *M. tuberculosis* culture-positive strains, 5 out of 6 rifampicin resistant strains were detected by the PCR-UHR assay. The specimen giving a susceptible result by the PCR-UHG assay but resistant by culture, did not contain a mutation in the *rpo*B region. As stated by the authors, the most cost-effective application of this assay would be at reference laboratories receiving large numbers of specimens since this would decrease the cost of the test per specimen.

2.4 Solid phase hybridization assay

The Line Probe Assay (LiPA) (Innogenetics N.V., Zwijndrecht, Belgium) is a commercial test for the rapid detection of the *M. tuberculosis* complex and rifampicin resistance. LiPA is based on the hybridization of amplified DNA from cultured strains or clinical samples to ten probes encompassing the core region of the *rpo*B gene of *M. tuberculosis*, which are immobilized on a nitrocellulose strip [21]. 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 occur, the mutation is present. In the original study by De Beenhouwer et al. [21], LiPA results matched classical testing in 65 out of 67 specimens. Further evaluation of this assay by Cooksey et al. [22] with a collection of 51 rifampicin-resistant strains gave an overall concordance of 90.2% with phenotypic susceptibility testing. Rossau et al. [23] evaluated 107 *M. tuberculosis* isolates with known *rpo*B sequences, 52 non-*M. tuberculosis* complex strains, and 61 sensitive and 203 resistant clinical isolates, and their results indicated that the probe was 100% specific for the *M. tuberculosis* complex; no discrepancies were observed with the results of nucleotide sequencing. Furthermore, all strains sensitive by in vitro susceptibility testing were identified correctly, and among the resistant strains only 2 % yielded conflicting results. More recently, Gamboa et al. [24] evaluated the LiPA with 59 *M. tuberculosis* culture-positive specimens (most of respiratory origin) and found a concordance of 98.3% with the DST of the isolated strains. As with all commercial methods however, the cost of the kit may limit its use in many developing countries.

2.5 Emerging new technologies for rapid detection of MDRTB

2.5.1 DNA Microarrays

One recent approach for genotypic detection of resistance is based on hybridization of amplified DNA to high density oligonucleotide arrays on a glass miniaturized support giving the possibility to examine large amounts of sequence in a single hybridization step. Gingeras et al. [25] designed an array to determine the specific nucleotide sequence of a 705-bp fragment of the *rpo*B gene, detecting rifampicin resistance in 44 clinical isolates evaluated. Additionally, they were able to simultaneously genotype and speciate non-tuberculous isolates. More recently Troesch et al [26] have used this same technology for *Mycobacterium* species identification and rifampicin resistance detection using two sequence databases. By hybridizing fluorescent-labelled amplified genetic material from mycobacterial colonies, 27 different species were detected, as was rifampicin resistance in 15 evaluated strains. To circumvent some of the limitations of the original technique such as high cost, complexity and difficulty for interpretation, Head et al. [27] have reported the use of a moderate density array, allowing efficient and easy to interpret sequence information. They detected and characterized mutations in nine resistant isolates evaluated, while no mutation was found in the one susceptible strain tested. The potential to include additional probes for other drugs in the same solid support would be an added advantage to the technique; however, there is still the concern about the costs, complexity and requirement of skilled personnel, that hampers the application of these technologies in standard mycobacteriology laboratory settings.

2.5.2 Reporter systems

Two main approaches for the use of reporter systems have been proposed. Firstly, Cooksey et al. [28] described the construction of plasmids containing the firefly luciferase gene that were used to transform an avirulent strain of *M. tuberculosis*. The production of light was then detected 48 hours after these transformants had been incubated in the presence or absence of drugs. Later on, Arain et al. [29] employed reporter strains of *M. tuberculosis* and *M. bovis* BCG endogenously expressing luciferase to test the activity of rifampicin and isoniazid. They also developed a standardized system (Bio-Siv) for bioluminescence assay of several antimicrobials, including isoniazid, ethambutol, rifampicin, amikacin, streptomycin and ciprofloxacin, where MICs values correlated with conventional methods in

the BACTEC® system [30]. Similarly, Hickey et al. [31] have used an enhanced luciferase-expressing mycobacterium for evaluating antimycobacterial activity directly in mice. More recently, Shawar et al. employed recombinant strains of *M. bovis* BCG and *M. intracellulare* in a 96-well mini-tube format for detecting antimycobacterial activity in the extracts of natural products [32].

The other approach with reporter systems has been the use of phages as vectors to introduce the luciferase gene. For example, Jacobs and collaborators [33] cloned the luciferase gene into the genome of mycobacteriophages and expressed the gene in *M. tuberculosis*; DST was performed by assessing the production of light by viable phage-infected mycobacteria. The light was detectable minutes after infection. Cultivation of susceptible *M. tuberculosis* strains in the presence of rifampicin or isoniazid caused extinction of the light signal. This same group has modified the system using another phage (phAE88), which produces increased intensity and enhanced duration of the light signal, allowing detection of drug activity within one day [34]. A further modification allows detection of the emitted light with a custom-made Polaroid® film box, termed the Bronx box [35].

Reporter systems other than luciferase have also been described [36,37]. For example, the green fluorescence protein (GFP) of the jellyfish *Aequorea victoria* has been used as a reporter molecule. This reporter system does not require cofactors or substrates due to the intrinsically fluorescent nature of GFP. However, these alternative reporter systems have generally only been used for the screening of new compounds with activity against *M. tuberculosis* and not for clinical AST [38-40].

Although the reporter systems described above have shown in general good sensitivity and reproducibility, there is still the issue of the cost of implementation in endemic countries and at the level of the clinical mycobacteriology laboratory.

2.5.3 Miscellaneous genotypic techniques

Several other new genotypic techniques have been proposed for the rapid detection of drug resistance in *M. tuberculosis*: cleavase fragment length polymorphism (CFLP) [41], dideoxy fingerprinting (ddF) [42,43], hybridization protection assays [44-46], a method based on reverse transcriptase-strand displacement amplification of mRNA [47], RNA/RNA duplex base-pair mismatch assay [48], and DNA sequence analysis using fluorogenic reporter molecules (ie. molecular beacons) [49]. However, these techniques have not been extensively studied and have not been further validated with clinical isolates. Although they share the 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.

3. NOVEL PHENOTYPIC TECHNIQUES

Phenotypic techniques have generally relied on culture methods that require the visual detection of *M. tuberculosis* colonies for interpretation of results. Due to the slow growth rate of *M. tuberculosis*, these phenotypic methods require several weeks before completion. A novel group of phenotypic methods are being introduced that provide 'rapid' results by detecting earlier signs of mycobacterial growth using various technologies: measurement of metabolism with the aid of color indicators, detection of oxygen consumption, or early visualization of microcolonies.

3.1 Mycobacteria growth indicator tube (MGIT)

Among the commercial systems recently developed for the rapid detection of mycobacterial growth, the MGIT system (Becton Dickinson, USA) has been evaluated in several comparative studies for the early detection of *M. tuberculosis* and other mycobacteria [50-52]. The system was subsequently adapted for *M. tuberculosis* DST. The MGIT system consists of glass tubes containing a modified Middlebrook 7H9 liquid medium together with a fluorescence quenching-based oxygen sensor embedded at the bottom of each tube. When inoculated with *M. tuberculosis*, consumption of the dissolved oxygen will produce fluorescence when illuminated by a UV lamp. For DST, a set containing a growth control and drug-containing tubes is inoculated with the isolate under study, and after a period of incubation at 37°C, growth is compared in the drug-containing and control tubes allowing determination of susceptibility or resistance. Several evaluations have been reported of MGIT for the rapid detection of drug resistant *M. tuberculosis*. Reisner et al. [53] compared the reliability of MGIT for isoniazid and rifampicin DST with the BACTEC® system for 29 isolates. They reported a TAT of 3-8 days (median 6 days) for MGIT and 4-10 days (median 6 days) for BACTEC®, and obtained full agreement for isoniazid and for 28/29 isolates with rifampicin. In other studies, the MGIT system has compared fairly well with the proportion method on Löwenstein-Jensen (LJ) medium, agar-based medium, and BACTEC® [54-59], especially for rifampicin and isoniazid; however, further standardization is still needed

for ethambutol and streptomycin. No MGIT DST studies have been done with second-line antibiotics for *M. tuberculosis*. Very recently, an automated system using the MGIT tubes has been introduced by the manufacturer [60], which claims to reduce the time to detection and to facilitate the manipulation and reading of large numbers of samples. However, unnecessary introduction of this equipment would increase the cost of the procedure, preventing its use in laboratories with limited resources.

3.2 The PhaB assay

A new phenotypic culture-based DST, the phage amplified biologically (PhaB) assay, has been recently introduced [61]. This test 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 then determined after cycles of infection, replication and release in a rapidly-growing mycobacteria. When evaluated with 46 *M. tuberculosis* clinical isolates, the assay correctly identified susceptibility or resistance to rifampicin in 44 isolates (95.7%), and to isoniazid in 40 of 46 isolates (87%). Results were available within 3-4 days. The test could theoretically be performed directly on patient samples, reducing even further the time for drug susceptibility results. One limitation of the assay, however, is the specificity of the mycobacteriophage (D29) used in the procedure. This phage can infect other mycobacteria. This problem could be overcome by choosing an alternative bacteriophage or by altering the specificity of D29. Additional studies are needed to evaluate the performance of the test in different laboratory settings [62].

3.3 E-test

The E-test, another commercial system (AB BIODISK, Sweden), is based on determination of drug susceptibility using strips containing gradients of impregnated antibiotics. Strips containing the drug of choice are applied on the surface of an agar medium inoculated with the test strain; after a period of incubation, the minimum inhibitory concentration (MIC) is read from the point at which the ellipse (formed by the inhibition of growth) intersects the strip [63]. The test has been applied to a variety of difficult-to-grow microorganisms including rapidly growing mycobacteria [64-67]. Wanger & Mills have compared the E-test with the BACTEC® and agar proportion methods for testing the susceptibility of *M. tuberculosis* to four first-line anti-tuberculosis drugs and reported equivalent interpretive results

for all the strains evaluated [68,69]. Although the values were within ±2 $\log_2$ dilutions, agreement was found to be 93, 100, 90, and 94% for isoniazid, rifampicin, ethambutol, and streptomycin, respectively. Results were obtained within 5 to 10 days using a rather high inoculum, equivalent to a McFarland 3.0 standard. More recently, Hausdorfer et al. [70] have evaluated the E-test with the proportion method on LJ and BACTEC® for the same four drugs; of 81 isolates evaluated, 73 (90.1%) gave concordant results for the four drugs (69 susceptible and 4 resistant). The eight remaining isolates were susceptible by the proportion method, but resistant by the E-test, five resistant to ethambutol, two resistant for isoniazid and one resistant to both ethambutol and streptomycin. The authors concluded that due to the high rate of false resistance, the method could not be recommended for use in clinical laboratories. No further studies have been performed to clarify the usefulness of the E-test for the routine mycobacteriology laboratory. The cost of the strips is also a disadvantage.

3.4 Rapid metabolic tests

Tetrazolium salts have been used to study metabolism and viability in a number of microorganisms [71-73], and to measure toxicity for eucaryotic and procaryotic cells [74,75]. Yajko et al. [76] were the first to describe a colorimetric method based on an oxidation-reduction dye, Alamar blue, for the quantitative measurement of drug susceptibility in *M. tuberculosis*. The dye in the oxidized state is blue but is pink when reduced. The change is easily discernible visually, or can be measured spectrophotometrically or fluorometrically. For DST, a group of tubes containing dilutions of each antituberculous drug and a control tube without any drug are inoculated with the isolate under study. After a period of incubation (ie. 7, 10 and 14 days), the Alamar blue is added and the tubes again incubated for color development; those tubes supporting growth of the bacteria (ie. resistant to the drug) reduce the indicator, changing the color from blue to pink. Yajko et al studied 50 isolates of *M. tuberculosis* determining the MICs of isoniazid, rifampicin, ethambutol and streptomycin, and comparing the results with those obtained by the agar proportion method; interpretive agreement between the two methods was 98% for isoniazid, rifampicin and ethambutol and 94% for streptomycin. Collins & Franzblau [77] adapted the test to a microtiter format, and compared this format with the BACTEC® system for high throughput screening of compounds against *M. tuberculosis* and *M. avium*. They subsequently used the microtiter system in a laboratory in a low-income country to determine the MICs of isoniazid, rifampicin, ethambutol and streptomycin for 34 clinical isolates of *M. tuberculosis* [78]. The microtiter results were available within 8 days and had an overall

agreement of 93.6% (after re-testing 12 of 17 samples with discrepant results) with results obtained using the BACTEC® system. More recently, Palomino & Portaels [79] have evaluated a similar test using one critical concentration of each of the four drugs. Compared with the proportion method on LJ, they found an overall agreement of 97% for all four drugs (100% for isoniazid and rifampicin); results were available after 8 days of incubation.

Mshana et al. [80] and Abate et al. [81] have proposed a similar colorimetric system, which uses 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as the viability indicator, for the rapid detection of rifampicin resistance. In this case, the dye changes from yellow to blue, and this color change can also be visually differentiated. In a tube macro-method with Dubos broth, they studied 92 clinical isolates and obtained full concordance for their assay compared with the BACTEC® system. Additional evaluations show that this system can be successfully adapted for the study of first and second-line antituberculous drugs (Julieta Luna, personal communication).

3.5 Microcolony detection method

Some studies have evaluated a method of microcolony detection on solid media. When inoculated on a thin layer of agar, such as Middlebrook 7H11 in a Petri plate, mycobacteria form typical microcolonies easily detectable with a microscope. In evaluations for the rapid detection and diagnosis of *M. tuberculosis*, this method has compared favorably with conventional culture on LJ [82-84]. When applied as a rapid method for *M. tuberculosis* DST, Schaberg et al. [85] found that microcolony detection produced shorter median TAT than conventional methods for 64 smear-positive (ie. 11 vs 62 days) and 133 smear-negative specimens (35 vs 72 days). All cases of single-drug and multi-drug resistance were correctly identified. Current evaluations of the microcolony detection method for rapid susceptibility testing of rifampicin, isoniazid and second-line drugs show a very good correlation with the proportion method. Microcolony detection therefore provides an alternative method for rapid DST in laboratories with limited resources (Jaime Robledo, personal communication); further evaluations of this low-cost methodology are necessary to assess its usefulness for clinical mycobacteriology laboratories in low-income countries.

3.6 Miscellaneous phenotypic procedures

Several other procedures have been proposed for the rapid detection of drug resistance in *M. tuberculosis*. For example, a bioluminescence assay

has been used to detect ATP produced by viable *M. tuberculosis* in the presence and absence of antibiotics. The bioluminescence assay was evaluated with the first-line drugs and gave results in 5-7 days with good correlation with the resistance ratio method and BACTEC® [86, 87]. Another rapid DST method for isoniazid and streptomycin involves measurement of mycolic acid levels using high performance liquid chromatography [88]; standardization of the assay was done with *M. tuberculosis* H37Ra and results were obtained in 3 to 4 days. However, no evaluation of this test has been done with clinical isolates of *M. tuberculosis*. Drug susceptibility testing has also been attempted by flow cytometry using *M. tuberculosis* labelled with fluorescein diacetate; the procedure was evaluated on 17 clinical isolates with ethambutol, isoniazid and rifampicin and the results were available after 3 days [89,90]. The new ESP culture system II (Accumed International, USA), which detects pressure changes resulting from the consumption or production of gas by growing mycobacteria, has also been evaluated for DST of the four first-line drugs [91]. The agreement of the ESP results with BACTEC® ranged from 93 to 100 % in an evaluation of 20 clinical strains and 30 challenge strains of *M. tuberculosis*; additional studies will be necessary to assess the performance of this new ESP system. As with other automated methods, this system has the disadvantage of requiring sophisticated equipment, which is only available from one commercial source, thereby limiting its wider application in developing countries.

4. CONCLUSIONS

Many new possibilities have arisen for detecting drug resistance in *M. tuberculosis* and for performing DST. These novel methods rely on new information concerning molecular mechanisms of drug resistance, or on new approaches in detecting mycobacterial growth. The first group of technologies, categorized as genotypic methods, has 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. Furthermore, the sophistication of some of these methods and their requirement for expensive equipment restrict their implementation to laboratories in developed countries or to reference laboratories with the necessary resources of equipment and personnel (Figure 1). The second group of technologies, categorized as phenotypic methods, is more diverse. Some of them, although being simple in their procedure, still require expensive equipment not always available in laboratories in TB-endemic countries. Others involve uncomplicated procedures that could be easily

implemented in routine mycobacteriology laboratories. However, these phenotypic methods require further careful evaluation and validation to obtain acceptable levels of sensitivity, specificity and reproducibility before they replace the current DST procedures.

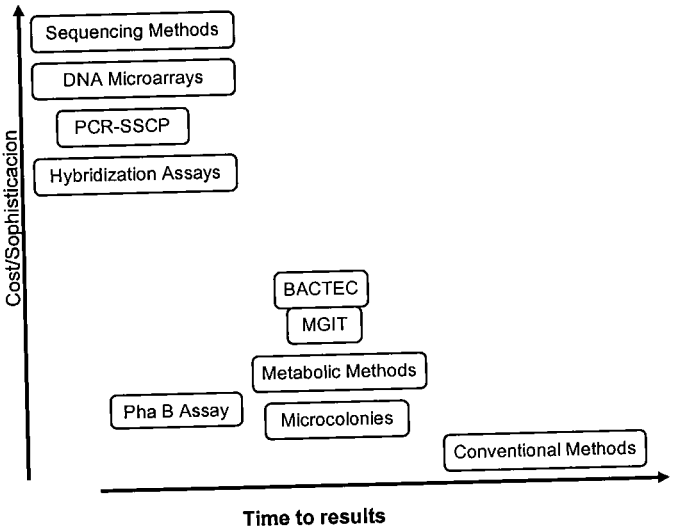

Figure 1. Comparisons of turnaround-time (TAT) versus cost and sophistication for various drug susceptibility testing methodologies. Each laboratory must consider their circumstances, their financial resources, and their expertise, before choosing the most appropriate technique for improving their detection of drug resistant *M. tuberculosis*.

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