LABORATORY METHODS FOR DIAGNOSIS AND DETECTION OF DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS COMPLEX WITH REFERENCE TO DEVELOPING COUNTRIES: A REVIEW

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ABSTRACT

Objective: To outline principles, advantages and limitations of the currently available laboratory methods for diagnosis and detection of drug resistance of Mycobacterium tuberculosis complex.

Data source: Published series of peer reviewed journals and manuals written on laboratory methods that are currently used for diagnosis and detection of drug resistance of Mycobacterium tuberculosis complex were reviewed using the index medicus, pubmed and medline search. Conventional bacteriological microscopy and culture, BACTEC, and molecular-based techniques were included. Basic principles, advantages and limitations of the cited techniques have been highlighted.

Conclusion: Conventional bacteriological microscopy and culture are usually used for diagnosis of tuberculosis (TB) particularly in developing countries. However, their limited sensitivity, specificity and delayed results make this provision inadequate. Despite the development of quicker and more sensitive novel diagnostic techniques, their complexity and high cost has limited their use in many poor-resource countries. Due to the rapidly growing TB problem in these countries, there is urgent need to assess promising alternative methodologies in settings with high disease prevalence.

INTRODUCTION

In developed as well as developing countries, the presumptive diagnosis of the infectious cases of tuberculosis (TB) is based on the identification of acid-fast bacilli in a sputum specimen. The technique is fairly rapid but has limited sensitivity and specificity(1). Culture is the gold standard for the diagnosis of TB. The culture of sputum or other specimens allows confirmation of cases and also facilitates species identification. Low numbers of mycobacteria that cannot be detected by microscopy are detected by culture and drug susceptibility assays can be performed when culture is available. However, culture takes a long time before results are available(1). With the re-emergence of TB and the increasing rates of outbreaks of multi-drug resistant tuberculosis (MDR-TB), newer methods for diagnosis and detection of mycobacteria have been introduced. These include phenotypic-based techniques such as BACTEC systems(2,3) and molecular based methods including Polymerase chain reaction (PCR)(4,5). Although these methods are fast and results can be obtained within two to three days, the high operational cost component and the requirement for highly qualified manpower, make them unsuitable for many laboratoires, and especially those with limited resources. While there is a need to improve the existing conventional diagnostic facilities, the increase in TB cases due to the HIV/TB pandemic and inadequate conventional procedures currently in use, call for urgent assessment of promising alternative methodologies especially in settings with high prevalent disease. This review outlines the basic principles and limitations of available methods for laboratory diagnosis, identification and detection of drug resistance of Mycobacterium tuberculosis (MTB) complex which include M. tuberculosis, M. africanum, M. microti, M. bovis, M. canetti, and M. bovis BCG, a group of strains that usually cause TB(6).

DIAGNOSTIC METHODS

For the bacteriological diagnosis of TB, conventional methods for smear microscopy and culture are used. However, because of the rapidly growing TB problem in the developing world the existing provision is inadequate.

MICROSCOPY

Diagnosis by microscopy is carried out either after staining with carbol-fuchsin using the Ziehl-Neelsen (ZN) method or after staining with auramine O or rhodamine, the most frequently used stain among those methods based on fluorescence microscopy(6,7). The procedure is of intermediate complexity and gives results within a few hours. However, it fails to detect low numbers of mycobacteria present in a specimen and is also not specific for the MTB complex. Microscopy carried out using the ZN method can detect approximately $10^4$ bacilli per ml of sputum(1), with a sensitivity of between 60% and 70% of
culture positive sputum specimens(8). Fluorescent microscopy is more rapid and more sensitive than the ZN method(9) but because of the cost component of the equipment needed, it is rarely used in most developing countries. Although microscopy is of intermediate complexity and gives results within a few hours, it cannot detect drug resistant strains and only a limited number of sputum samples can be handled on a daily basis if the method is carried out meticulously. Furthermore, factors that are related to the HIV/TB pandemic including large numbers of individuals being screened and poor quality sputum specimens further make the diagnosis by microscopy in these countries inadequate.

CULTURE

The culture allows confirmation of cases and also facilitates species identification. With this method, very low numbers of mycobacteria can be detected and susceptibility assays performed. However, culture techniques require more skilled personnel and additional equipment. Thus, culture methods are not normally available within most diagnostic facilities except in central reference laboratories and research institutions. Below is a description of some of these methods.

CONVENTIONAL METHODS

Egg-based media such as Lowenstein-Jensen (L-J), agar-based media including Middlebrook (7H10/7H11) and liquid media such as Kirchner and Dubois broth is commonly used(6). Because of the slow growth of the tubercle bacilli (generation time 18-24 hours), mycobacteria isolated from specimens from non sterile body sites such as sputum, are easily overgrown by more rapidly dividing organisms including bacteria and fungi.

Procedures which involve digestion and decontamination of specimens before culture process and which do not interfere with the viability of the mycobacteria have been described. The most commonly used is the N-acetyl-L-cysteine (NALC)/sodium hydroxide (NaOH) technique(6). Other protocols use the modified Petroff’s NaOH technique(10,11). Slopes of LJ are then inoculated and incubated at 37°C and checked once a week until either growth appears or slopes are declared negative at the end of the eighth week of incubation. This leads to delays in obtaining results(1).

NEW METHODS

New culture methods include: (a) The radiometric semi-automated BACTEC 460TB system (Becton Dickinson, Heidelberg, Germany). This technique is based on the release of radiolabeled CO₂ in liquid cultures as an indicator for cell metabolism and growth; (b) The fully automated systems such as MB/BacT system (Organon Teknica, Turnabaut, Belgium)(12), ESP culture system 11(AccuMed, Chicago, Ill.) (13), the BACTEC9000 MB system (Becton Dickinson) (2) and the BACTEC Mycobacterium Growth Indicator Tube (BACTEC MGIT 960 system: Becton Dickinson)(3). These systems utilize a colorimetric sensor and reflected light to continuously monitor the CO₂ concentration in the culture medium and; (c) manual systems such as the fluorometric MGIT (Becton Dickinson) (14) and MB-Radox (Boi et, Dreieich, Germany)(15). With these methods bacterial growth can be obtained within 2 to 3 weeks.

Several studies have been carried out to identify a "better gold standard" than conventional culture. A study comparing three methods of LJ, BACTEC 460 TB, and MGIT for isolation of MTB indicated that there was no significant difference between them(16). However, MGIT had the advantage of a shorter incubation period and low labour intensity (16). Another study that compared MB/ BacT and BACTEC 460 TB systems showed a lack of sensitivity with smear-negative patients when using the MB/BacT system(12). In a study by Tortoli et al.(3) the contamination rate was higher with the BACTEC MGIT 960 system than with the radiometric BACTEC 460 TB system.

It is important to note that all standard culture methods including conventional ones, when carried out meticulously, will allow isolation of all species of mycobacteria which require application of specific identification procedures as will be elaborated later in this review.

MOLECULAR AMPLIFICATION-BASED DIAGNOSTIC METHODS

Considerable effort has been invested in the use of the polymerase chain reaction (PCR) technique for the diagnosis of TB due to its ability to specifically amplify minute quantities of nucleic acid targeting either DNA or RNA. PCR amplifications involve extraction of DNA from clinical specimens. Target DNA is incubated in a buffer that usually consists of magnesium chloride, primers designed to bind specifically to the target sequences of the DNA, Taq polymerase enzyme and nucleotides.

The main problem with PCR assays is the high risk of obtaining false positives caused by contamination with carry-over amplicons(17) and false negatives mostly due to Taq polymerase inhibitors(18). However, carry-over contamination can be controlled by either of the following two steps: a) Incorporating dUTP in all PCR products by substituting dUTP for dTTP, or by incorporating uracil during synthesis of the oligodeoxyribonucleotide primers; b) Treating all subsequent fully pre-assembled starting reactions with uracil DNA glycosylase (UDG), followed by thermal inactivation of UDG(17,18). In addition, adopting and enforcing a three-room strategy has been recommended for reducing the problem of carry-over contamination of PCR products. The presence of inhibitors can be detected by spiking part of each sample analysed with MTB target DNA(18).

A variety of PCR-based techniques for detection and
identification of mycobacteria have been developed, most of them in commercial kits, and are now being evaluated. They include: a) The Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test (AMTD; Gen-Probe, USA). This identifies MTB from respiratory samples and subsequently amplifying reverse transcribed rRNA, and the product is detected with a specific chemiluminescent probe. b) The COBAS AMPLICOR PCR amplifies part of the 16S rRNA gene and the product is detected colorimetrically. c) The ligase chain assay (LCx; Probe System MTB; Abbott, USA) uses the gene encoding protein antigen b as the target template. d) Strand displacement amplification (BD robe Tec-SDA) uses a Kleenow fragment of DNA polymerase I to amplify DNA products from a DNA template of rRNA. e) LiPA MYCOBACTERIA (Innogenetics, Zwijndrecht, Belgium), is based on the hybridization of the 16-23 ribosomal spacer region PCR products to probes bound to a nylon strip membrane, and binding is identified colorimetrically.

The results for all these different techniques have indicated comparable sensitivity and specificity(19). However, the specificity of AMTD for the detection of MTB has been questioned due to false positive results for patients with pulmonary M. kansasii and M. avium infections(20). Furthermore, despite these techniques being rapid, the majority of them are less sensitive than culture. Thus, they are usually used alongside conventional culture techniques(4, 19). Consequently this increases the cost of the test, a limitation for routine application of these techniques in most developing countries.

SPECIES IDENTIFICATION METHODS

Since the procedures used for the isolation of mycobacteria are not usually species specific, a number of techniques for identifying mycobacteria at species level have been established. They include classical (conventional) methods, rapid identification methods and molecular-based methods.

CONVENTIONAL METHODS

Morphology: Colonial morphology on solid media provides preliminary identification of different mycobacterial species. On LJ media, colonies of MTB complex, for instance, appear rough and dry with buff colour(6). Microscopic appearance has been used, to a lesser extent, to differentiate species of mycobacteria. Although cord formation is considered as a strong indicator for strains of MTB complex(6,21), similar characteristics have been identified in other mycobacterial species including M. avium complex, M. gordoneae, M. chelonae, and M. marinum, when grown in liquid media(22).

Growth culture: Growth characteristics have also been used to identify mycobacterial species. These include rate of growth, preference for aerobic or microaerophilic environments, preference for growth on L-J slopes containing pyruvate or glycerol, pigment production, niacin production, susceptibility to 5mg/l thiophene carboxylic acid hydrazide (TCH), 500 mg/l p-nitrobenzoate (PNB), 15 mg/l cycloserine, and 66 mg/l pyrazinamide (Z), and ability to reduce nitrate to nitrite and to hydrolyze Tween 80(6).

Temperature range: Although the majority of mycobacterial species grow at 37°C, some species show optimal growth at different temperature ranges(6). Members of the MTB complex best grow at 37°C, do not grow at 25°C and 42°C, do not produce pigment, are inhibited by PNB and visible growth is not apparent within three days of subculture(6,11,23).

The subspecies of the MTB complex may be distinguished by further tests as indicated in Table 1(6).

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Oxygen preference</th>
<th>Growth on pyruvate</th>
<th>Growth on glycerol</th>
<th>TCH</th>
<th>Cycloserine</th>
<th>PZA</th>
<th>Nitrate reduction</th>
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<tr>
<td>Classical</td>
<td>A</td>
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<td>R</td>
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<tr>
<td>Asian</td>
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<td>Africanum I</td>
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<td>Bovis</td>
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A = aerophile; M = micro-aerophile; G = glycerol; P = pyruvate; R = resistant; S = sensitive; + = positive; - = negative; v = variable; PZA = pyrazinamide; TCH = thiophene carboxylic acid hydrazide.

Classical identification procedures require up to three weeks following culture before the results are available.

RAPID IDENTIFICATION METHODS

High performance liquid chromatography (HPLC) identifies mycobacteria by the mycolic acid content of their cell walls. This method has the advantage of detecting and identifying mycobacterial mycolic acids in a clinical specimen after labelling the mycolic acids with a fluorescent chromophore(24). HPLC has facilitated separation of M. bovis (BCG) from MTB and M. bovis(25). However, HPLC requires the use of expensive equipment and this makes it only viable in laboratories with ample funding. Gas-liquid chromatography (GLC) of shorter-chain fatty acid esters and mycolic acids has provided an unequivocal identification of mycobacterial species including MTB within a shorter period than conventional culture(26).

Preliminary identification of IMTB complex can also be carried out with the radiometric BACTEC p-nitro-α-acetamino-β-hydroxypropiofenone (NAF) test which evaluates specific inhibition of growth of MTB complex by NAP, a precursor in the synthesis of chloramphenicol(27). However, the results obtained are considered preliminary since false positives are observed(28).
MOLECULAR-BASED SPECIES IDENTIFICATION METHODS

With the re-emergence of TB due to MTB and some nontuberculous mycobacteria such as *M. Kansasii* and *M. avium* complex, species identification has now become more significant than it was before. Sequencing of the 16S rRNA gene, based on the species-specific nucleotide sequence of the hypervariable region of this gene, has allowed identification of different species of mycobacteria(29). The technique is specific but costly and labour intensive.

Alternative approaches to the rapid identification of MTB have focused on the use of species-specific nucleic acid probes. An oligonucleotide probe, based on the 16S rRNA gene sequence of MTB is now commercially available (AccuProbe, Gen-Probe Incorporated, San Diego, USA). The probe allows the identification of the species of the MTB complex. However, at least one million bacilli are required in a sample for a reliable identification. Consequently, this limits the application of these probes in the identification of pathogenic mycobacteria from clinical samples(30). With the completion of sequencing of the MTB genome(31) and the subsequent development of highly sensitive probes, some of these problems may be resolved.

Gene targets have been used in broad-range consensus PCR assays for the identification of mycobacteria. The PCR restriction enzyme assay (RRA) based on the amplification of a fragment of a hsp65 gene encoding a 65-kDa heat shock protein that is present in all mycobacteria, followed by two separate restriction enzyme digestions has been used successfully to identify different species of mycobacteria(32). The 65-kDa protein contains epitopes that are unique as well as epitopes that are common to various species of mycobacteria(33). Thus, the conserved nature of hsp65 gene allows differentiation of mycobacteria(32). Furthermore, sequence analysis of amplified rpoB DNAs has been shown to efficiently identify clinical isolates of mycobacteria in parallel with traditional culture methods and, as a supplement to 16S rDNA gene analysis (34). However, these sequences are identical for all members of the MTB complex(33). Thus, a battery of classical biochemical tests must be performed for a specific identification. Another technique for species identification of MTB has been described. The method based on the sequence of mtp40 has been identified as part of the mtpA gene. This gene encodes a phospholipase C region of MTB and carries a particular mutation at position 169 of the gene conferring pyrazinamide resistance(33). Although originally thought to be specific for MTB(36), subsequent studies have indicated that mtp40 sequence is not present in all strains of MTB(37).

SEROLOGICAL TESTS

Several studies based on the detection of antibodies to a variety of mycobacterial antigens or bacteria-derived antigens have been carried out but none has proved reliable so far. Lack of sensitivity and specificity are the main problems encountered when using immunological techniques. However, their use is becoming increasingly important in the diagnosis of child TB(38). Cytokine-based assays to determine the relationship between taberculin skin test response and cytokine profile have recently shown encouraging results, thus allowing epidemiological studies on the immunity to TB in humans(39).

METHODS FOR THE DETECTION OF DRUG RESISTANCE

Conventional methods: There are three well-recognised classical methods for antituberculosis drug susceptibility testing. a) The absolute concentration method: In this method, media containing several sequential two-fold dilutions of each drug are used and resistance is indicated by the lowest concentration of the drug which will inhibit growth (on LJ slope showing less than 20 colonies at the end of 4 weeks incubation). b) The resistance-ratio (RR) method: This is a variant of the absolute concentration method and is defined as the minimum inhibitory concentration (MIC) of the test strain divided by the MIC of the standard susceptible strain H37Rv in the same set of tests. A RR of 2 or less defines drug susceptibility, a RR of 4 is considered low level or intermediate drug resistance while a RR of 8 or more is considered evidence of high drug resistance. c) The proportional method: This is the most commonly used method where the ratio between the number of colonies growing in the drug-free medium indicates the proportion of drug resistant bacilli present in the bacterial population. From the bacterial colony counts the proportion of mutants resistant to the drug concentration tested is determined and expressed as a percentage of the total number of viable colony forming units in the population. High and low inocula are included and serve as controls(6,23,40).

Although useful data have been gathered as a result of using these three methods, problems of standardization remain unresolved. Difficulties include establishing appropriate inoculum size (with the exception of the proportional method), the stability of drug compounds in different culture media and the reliability of results obtained when testing some of the drugs(41). In addition, these methods take up to 4 weeks after culture before the results are obtained.

Rapid phenotypic methods: Increasing rates of drug resistance and outbreaks of MDR-TB globally have facilitated development of rapid methods for detection of antimycobacterial resistance. They include phenotypic methods such as the Bactec 460T Bactec system(41). The phage amplified biologically (PhaB) assay is based on the detection of phages (mycobacteriophage D29) which have successfully infected viable mycobacteria that have previously been exposed to antituberculosis drugs(42). After replication in the mycobacteria, the
mycobacteriophages are released and detected by their ability to infect and lyse the fast growing M. *megatidis* on an agar plate by forming plaques in a lawn of bacterial growth. The number of plaques present is directly proportional to the drug resistant mycobacteria. This method was developed initially for the detection of isoniazid (H) and rifampicin (R) resistance but can be used in the diagnosis and susceptibility testing of MTB organisms in a patient sample or from an isolate(43). A study has shown that in addition to comparable results with genotypic and conventional methods, the PhoB assay has additional advantages, namely, simplicity and low cost(44).

Other phenotypic methods for detection of drug resistance have been assessed. A method using insertion of a firefly luciferase gene into the genetic material of a virus (luciferase reporter phage) has been described. This technique is based on the production of quantifiable light upon infection with viable mycobacteria which is subsequently detected with a Polaroid film box, the Bronx Box(44). Light is not observed in mycobacteria treated with active antimicrobials.

A colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for the rapid detection of rifampicin-resistant MTB in clinical specimens and isolates has also been developed(45). For its wider application, the technique needs further evaluation.

The main advantage with most of the phenotypic methods is that unlike the molecular detection systems, one need not be very knowledgeable about the genes encoding the drug target and the respective mutations producing resistance.

**MOLECULAR-BASED TECHNIQUES**

The possibility of predicting drug resistance of MTB rapidly from specific genomic mutations has recently attracted increased attention(46,47). However, a number of limitations including characterisation of genes with mutations which confer resistance and their relative significance still hamper development of effective methods for the detection of resistance to the commonly used drugs. Progress in the understanding of the basis of resistance to rifampicin (R) has facilitated the development of molecular tests for the detection of drug-resistant TB.

Several genotypic techniques for rapid detection of resistance to different drugs are currently being evaluated. Those designed for the detection of resistance in MTB include the use of Acil restriction enzyme digestion of a polymerase chain reaction (PCR) and amplified KatG and InhA fragments for the prediction of isoniazid (H) resistance(46). Apart from rifampicin, the complex nature of resistance to H(46) and other antituberculosis drugs has limited the development of new techniques in this area. However, since MDR-TB is defined as resistance to at least H and R(23) and most strains that are resistant to R are also resistant to H, resistance to R serves as a surrogate marker for MDR-TB(47).

A PCR based single-strand conformation polymorphism (SSCP) technique that allows rapid detection of single base changes in PCR products without sequencing of the rpoB gene has been described(47,48). It involves post amplification analysis of mutations in which the two amplified DNA strands are separated by denaturation and cooled to allow the separated DNA to fold into characteristic conformation. The products are analysed by gel electrophoresis and mutation is indicated by the difference in electrophoretic mobility(41).

Commercial kits are available for the detection of rpoB gene point mutations associated with R resistance. They include INNO-LiPA Rif. TB (Innogenetics, Zwijndrecht, Belgium) and MisMatch Detect II (Ambion, Austin, TX). INNO-LiPA Rif. TB is a reverse hybridization based line probe assay consisting of one oligonucleotide probe for the detection of MTB complex strains and nine probes designed to detect nucleotide changes in the relevant part of the rpoB gene(5). The MisMatch Detect II is based on the non-isotopic RNase cleavage of RNA/RNA duplex at any position of base mismatch and analysis of RNase treatment reaction by agarose gel electrophoresis. This method has shown high positive predictive values for the detection of rpoB gene mutations(49). Although these methods have the combined advantage of being rapid and can be done on primary specimens and cultures, studies done in conjunction with the conventional culture methods have indicated that some resistant strains are missed(5,43).

Characterization of mutations by automated DNA sequencing of the rpoB gene has been widely used for rapid detection of rifampicin resistant MTB(50). Micellar beacons which are single stranded nucleic molecules with a stem-and-loop structure have been used in the detection of resistance in MTB(51). The probes recognize and hybridise with specific nucleic acids in homogeneous solutions and also undergo a spontaneous fluorescent conformational change upon hybridization to their targets. Only perfect complementary targets elicit this response as hybridization will not occur when the target contains a mismatched nucleotide or a deletion(52). Although these methods are fast and results can be obtained within 2 to 3 days, the high operational cost component and the requirement for highly qualified manpower, make them unsuitable for many laboratories.

**CONCLUSION**

In conclusion, in spite of the development of faster and more sensitive techniques for the diagnosis and typing of TB complex strains, their complexity and high cost has limited their use in many low resource laboratories. When available, these “modern” methods are either found in national reference and research laboratories where the activities include a fundamental research component or in private institutions where a cost component may not be prohibitive. While there is a need to improve the existing conventional diagnostic facilities, the urgency of the situation demands urgent assessment of promising alternative methodologies for diagnosis and detection of
drug resistance of MTB complex strains under high prevalence conditions. This will provide a true picture of sensitivity, specificity, positive, and negative predictive values as well as their applicability. Transfer of technology and capacity building in local institutions will also be ensured. Furthermore, despite the many limitations in introduction of new technology to routine setting in most developing countries, the enormous diagnostic potential justifies the expectation that techniques based on newly-developed technologies will play an important and fundamental role in combating the rising rates of TB, and in particular MDR-TB. Genotypic assays for detection of mutations within the rpoB gene encoding the RNA polymerase $\beta$ subunit associated with rifampicin resistance hold promise as rapid and simple tests for the identification of most rifampicin resistant strains(5). The application of such techniques will be feasible when implemented at a national or regional level. Finally, quality control and quality assurance aspects need to be appropriately addressed before implementation of new technology is carried out.

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