Laboratory diagnosis of nontuberculous mycobacteria

Akos Somosković, Judit Mester, Yvonne M. Hale, Linda M. Parsons, Max Salfinger, MD*

aDepartment of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA
bDepartment of Respiratory Medicine, School of Medicine, Semmelweis University, Budapest, Hungary
cKorányi National Institute for Tuberculosis and Respiratory Medicine, Budapest, Hungary
dWadsworth Center, New York State Department of Health, Albany, NY, USA
eBureau of Laboratories, Florida Department of Health, Jacksonville, FL, USA
fDepartment of Biomedical Sciences, School of Public Health, University at Albany, Albany, NY, USA
gDepartment of Medicine, Albany Medical College, Albany, NY, USA

Even in the twenty-first century, mycobacterial diseases continue to cause serious public health problems. Although the number of cases of tuberculosis are decreasing in the United States, nontuberculous mycobacteria (NTM) are being recovered with increasing frequency from both immunocompromised (ie, HIV infected, cystic fibrosis patients) and immunocompetent patients. The clinical significance of these isolates has to be determined in each case because NTM are prevalent in the environment [1]. NTM were not traditionally considered a threat to public health, as person-to-person transmission occurs rarely if at all, yet these organisms can produce serious morbidity. In addition, cases of mycobacterial disease are becoming more difficult to diagnose or treat, especially when fastidious NTM or drug-resistant strains are involved [2,3].

Because of the increasing number of immunocompromised patients, immigrants, refugees, patients in congregate settings, and patients with drug-resistant disease, a wide array of clinical and public health specialists are required. The modern mycobacteriology laboratory plays a critical role in the laboratory diagnosis of tuberculosis and also diseases caused by NTM. These services must be accelerated and expanded not only in response to the changes in patient populations, but also to fulfill the need for shorter turnaround times that result in savings of limited health-care resources.

The most urgent questions that need to be addressed rapidly by the mycobacteriology laboratory include: (1) are tubercle bacilli or NTM involved?; (2) if NTM are involved, based on the existing clinical information (clinical, radiographic, and histological appearance, site of the disease, and source of specimen) and laboratory findings, does the isolated and/or identified NTM have any clinical significance?; (3) if a clinically significant NTM is involved, are efforts made to ensure a rapid identification?; and (4) if Mycobacterium avium complex, M. kansasii, M. fortuitum, M. abscessus, or M. chelonae are identified as clinically significant isolates, are susceptibility test assays performed according to standard procedures?

Twenty-five years ago, the Mycobacterium genus comprised only some 30 species. At present, it encompasses close to 100 [4]. The most recently described NTM species that are involved in pulmonary disease are listed in Table 1 [5–18]. Previously, the identification of NTM seemed to be feasible using a panel of cultural characteristics and biochemical tests [3]. The recent plethora of newly described species poses, however, an additional challenge for
the clinical mycobacteriology laboratory to provide accurate and timely services as conventional phenotypic tests are unacceptably time-consuming, less accurate, laborious, require special expertise, and often, after considerable effort, are not capable of identifying the NTM species at all [19]. Furthermore, some of the more recently described species (eg, *M. haemophilum, M. genavense*) require special growth conditions necessitating rarely used special media, as well as an exquisite collaboration between the clinician requesting the test and the laboratory professional performing the test.

The mycobacteriology laboratory has evolved through several phases in its effort to answer the previously outlined questions and challenges accurately and rapidly: from using radiometric growth detection including susceptibility testing in the early 1980s to using nucleic acid probes in the late 1980s, nucleic acid amplification (NAA) assays in the mid-1990s, and now DNA sequencing. This article summarizes the recent improvements in laboratory diagnosis and susceptibility testing of NTM in order to provide ideas about what the clinicians can expect from these new methods and how they can assess the performance of these laboratory services.

### Determination of the clinical significance of specimens

#### The relationship between the physician and the laboratory

In recent years, there has been a growing body of new and exciting methods in mycobacteriology, but there is no single test that can stand-alone for the identification of all mycobacteria [4,20]. Most importantly, complementary techniques should be used to generate complete and rapid information. The decision about which tests should be performed in-house and which sent to a reference laboratory should be made by the laboratory director based on the community to be served and the available resources, as well as in consultation with infectious disease professionals, pulmonologists, or other physicians involved. With this partnership, the physicians will then share the responsibility for the quality and the timeliness of the laboratory results.

In addition, laboratory test results should always be correlated with the patient’s clinical presentation, and radiographic and histological findings, and the clinician should notify the laboratory when results are inconsistent. An established and ongoing professional relationship between clinicians and the laboratory enables the recognition of inaccurate results earlier, and therefore may minimize any potential harm to the patient. This interaction becomes even more important when NTM are isolated because many of these species may cause human diseases, but many can also be found in the environment [1]. Although a specialized laboratory should be able to provide a precise species identification for most NTM isolates causing human diseases, the distinction between pathogen and saprophyte is not always clear-cut for isolates from a given individual.

Laboratory results alone are therefore not enough to dictate a particular strategy in the patient’s care, and a careful clinical correlation is necessary in determining the clinical significance of the specimen and making the correct diagnosis. Health care providers and laboratory staff need to communicate and cooperate to bridge any gap between them. Only when clinicians and laboratories work together can clinical outcomes be optimized [20,21].

#### Quality testing requires quality specimen

Accurate, rapid microbiological diagnosis of tuberculosis (TB) and other mycobacterial infections begins with proper specimen collection and rapid transport to the laboratory. To ensure collection of the best possible specimen, the health care worker has to be properly trained, and the patient provided with clearly presented and fully understood instructions for sputum and other specimen collection. The clearly labeled specimen must be transported to the laboratory quickly because the quality and turnaround time of some testing, such as acid-fast bacilli (AFB) smears and nucleic acid amplification, would be

<table>
<thead>
<tr>
<th>Year</th>
<th>Species</th>
<th>Reference</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1993</td>
<td><em>M. celatum</em></td>
<td>[6]</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td><em>M. genavense</em></td>
<td>[7]</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td><em>M. intermedium</em></td>
<td>[8]</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td><em>M. interjectum</em></td>
<td>[9]</td>
<td>Slow</td>
</tr>
<tr>
<td>1995</td>
<td><em>M. branderi</em></td>
<td>[10]</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td><em>M. mucogenicum</em></td>
<td>[12]</td>
<td>Rapid</td>
</tr>
<tr>
<td>1996</td>
<td><em>M. hodleri</em></td>
<td>[13]</td>
<td>Rapid</td>
</tr>
<tr>
<td></td>
<td><em>M. lentiflavum</em></td>
<td>[14]</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td><em>M. triplex</em></td>
<td>[15]</td>
<td>Slow</td>
</tr>
<tr>
<td>1997</td>
<td><em>M. heidelbergense</em></td>
<td>[16]</td>
<td>Slow</td>
</tr>
<tr>
<td>2000</td>
<td><em>M. heckeshornense</em></td>
<td>[17]</td>
<td>Slow</td>
</tr>
<tr>
<td>2001</td>
<td><em>M. immunogenicum</em></td>
<td>[18]</td>
<td>Rapid</td>
</tr>
</tbody>
</table>
amplifiable DNA or RNA from NTM) or sterile bronchoscopes that contain residual contaminants may occur. There is evidence that biopsy specimens that show necrotizing granulomas, non-necrotizing granulomas, poorly formed granulomas, or acute inflammation are optimal for mycobacterial growth detection. But biopsy specimens showing only fibrotic or hyalinized granulomas, nonspecific chronic inflammation, reactive or reparative changes, malignancy, or no significant abnormalities are inappropriate for mycobacterial culture and staining [24]. Aerosol-producing procedures should be performed in a way that ensures the safety of the health care worker during collection [25]. Most respiratory specimens will contain microorganisms other than mycobacteria; therefore, the specimen has to be refrigerated if a delay should occur, or, otherwise, overgrowth of more rapidly growing contaminants may occur.

Improper specimen collection can seriously hamper the determination of clinical significance of specimens. The best examples of this significant problem are the use of either nonsterile bronchoscopes (rinsed with nonsterile or nonfiltered water that contained NTM) or sterile bronchoscopes that contain residual amplifiable DNA or RNA from M. tuberculosis [26,27]. To avoid false-positive culture or amplification results from “Mycobacterium contaminated” bronchoscopes, it is recommended that the instrument be rinsed with sterile or filtered water, and for amplification tests, a sterile prewash of the bronchoscopes be performed and analyzed along with the actual clinical specimen [26,27]. Additional quality control procedures should also be developed in each health care facility to include visual inspection of the bronchoscope, regular testing for instrument integrity, maintenance, and surveillance for bronchoscopy related outbreaks or pseudo-outbreaks [28].

Detection and identification methods for NTM

Acid-fast microscopy

Acid-fast microscopy is the fastest, easiest, and least expensive tool for the rapid identification of patients with mycobacterial infections [20,22]. Although the specificity of acid-fast microscopy is excellent (all mycobacterial species are acid-fast), the sensitivity is not optimal, and this method is unable to distinguish within the Mycobacterium genus. The sensitivity of microscopy is influenced by numerous factors such as the prevalence and severity of tuberculosis or NTM disease, the type of specimen, the quality of specimen collection, the number of mycobacteria present in the specimen, the method of processing (direct or concentrated), the method of centrifugation, and most importantly, by the staining technique and the quality of the examination [22, 29–31]. Therefore, the overall sensitivity of microscopy is only between 22–65% [20,22,30]. It is recommended that a negative result should be reported only following the examination of at least 300 microscopic oil-immersion view fields (or equivalent fluorescent view fields). Therefore, when microscopy is performed correctly, it can be time-consuming and laborious. With large specimen loads, fatigue may lead to the reporting of a false-negative result, further decreasing the sensitivity of the assay. To facilitate proper patient management, all results should be reported to the physician within 24 hours of specimen collection or if an off-site laboratory is used within 24 hours after receipt of the specimen [22,32].

Presently, two types of acid-fast stains are used in clinical mycobacteriology laboratories. One is carbol fuchsin (Ziehl-Neelsen (ZN) or Kinyoun staining methods), and the other a fluorochrome (usually auramine or auramine-rhodamine). It is generally accepted that the fluorescent method should be given preference over the ZN and Kinyoun [20,33]. Results of a recent survey revealed, however, that the sensitivity and specificity of the ZN and fluorochrome methods are comparable, whereas Kinyoun’s cold carbol fuchsin method was found to be inferior to both the ZN and fluorochrome methods [34]. Moreover, it is often forgotten that fluorochrome stains may stain other bacteria damaged by antituberculous drugs at a higher rate than carbol fuchsin and lead to a false-positive result [30,35]. This possibility should be considered when the specimen is from a patient on therapy. It is also noteworthy that, because of a tendency toward false-positivity with fluorochrome staining, good laboratory practice requires that any doubtful and smear-positive results on newly diagnosed patients should be confirmed by ZN or by a second examiner [19,20,22,32].

Recently, in order to increase the efficacy of acid-fast microscopy, a model of a computer-directed automated microscope was constructed [36]. The automation and the time saving provided by this type of microscope can allow the examination of parallel
smeared from the same specimen, which could well increase the sensitivity. This model equipment still needs further evaluation, however, before it can be put into routine use.

Use of species-specific probes on AFB-positive sputum smears

Because the acid-fast stain cannot differentiate between *M tuberculosis* (MTB) complex organisms and NTM, a novel molecular biology approach using fluorescence in situ hybridization (FISH) appears promising. Two peptide nucleic acid probes targeting the 16S rRNA were developed to detect the MTB complex specifically and NTM in general in positive broth cultures [37,38] and potentially, for future routine use on AFB-positive sputum smears [39]. The FISH assay could be an asset in the peripheral laboratory’s armamentarium when serving communities with a substantial fraction of NTM diseases since no amplification equipment is needed. Although the method can readily differentiate between MTB complex and NTM, it is unable to identify the specific NTM present. In addition, the MTB-specific probe can give a weak false-positive signal with *M marinum* because there is only a single mismatch between the 16S rRNA sequence of this species and that of the MTB-specific probe [37]. Another drawback of the method is that the NTM-specific probe does not detect the presence of the relatively commonly isolated species, *M fortuitum*, *M flavescens*, and *M xenopi* [37]. These species all have more than one mismatch in the capture region of the NTM probe.

Direct nucleic acid amplification assays

Molecular tests, such as direct nucleic acid amplification (NAA) assays, can be used directly on the clinical specimens of patients suspected of having mycobacterial disease [40,41]. At present, the only commercial tests that offer the possibility to detect a NTM (*M avium*) along with MTB directly from clinical specimens and from the same amplification reaction are the Amplicor PCR assay (Roche Molecular Systems, Branchburg, NJ) and BDProbeTec (Becton-Dickinson Diagnostic Systems, Sparks, MD) strand displacement amplification technology [42,43]. But neither of these tests are approved yet for this testing aspect by the Food and Drug Administration in the United States. Recently, to improve the Amplicor polymerase chain reaction (PCR) assay and to detect the presence of a wide range of NTM, a *Mycobacterium* genus-specific capture probe was developed that can be used in conjunction with the pan-genus primers (targeting the 16S rRNA gene) and the MTB- and *M avium*-specific capture probes of the kit [42]. This genus-specific screening probe was tested with both respiratory and nonrespiratory specimens showing an initial sensitivity of 78.5% and a specificity of 93.5%. One limitation of this genus-specific probe is that it can give rise to “false-positive” results in the presence of clinically nonsignificant NTM in the specimen. To circumvent this problem, a second screening probe detects only the common potentially pathogenic (MTB complex, *M avium, M intracellulare*, *M kansasii, M xenopi, M malmoense, M leprae*) mycobacteria but not the clinically less significant ones [44]. The initial sensitivity and specificity of this screening probe were 89% and 93.9%, respectively [44]. Specimens positive with either of these screening probes can be further evaluated by hybridizing the amplicons to species-specific probes. These tests can be performed in as few as 6 – 8 hours on processed specimens, allowing same-day reporting of results.

Mycobacterial culture

It is clear that, although acid-fast microscopy and NAA are important adjuncts to the detection of mycobacterial infections, they are not an adequate criterion alone and must be followed by growth detection. Thus, culture for the presence of mycobacteria is still indispensable for the following reasons: (1) culture is more sensitive for the detection of mycobacteria than acid-fast microscopy, (2) growth of those NTM that are not covered by the presently available NAA assays is necessary for precise identification, (3) drug susceptibility tests require viable organisms, and (4) genotyping of particular cultured NTM (*ie, M avium, M xenopi, M malmoense, M abscessus, M chelonae*, etc) can be used for epidemiologic purposes and to rule out laboratory errors (cross contamination) [19,45,46].

Before culture, clinical specimens from nonsterile body sites must be subjected to a pretreatment involving homogenization, decontamination, and concentration. This procedure will eradicate more rapidly growing contaminants such as normal flora (other bacteria and fungi) but not seriously affecting the viability of the mycobacteria [22]. It is noteworthy, however, that the efficacy of these procedures is highly influenced by the time of exposure to the reagent used for decontamination, the toxicity of that reagent, the efficiency of centrifugation, and the killing effect of heat buildup during centrifugation [22]. There is evidence that even the mildest decontamination methods such as the widely used N-acetyl-L-cysteine/NaOH method can kill about 33% of the...
mycobacteria in a clinical specimen, whereas more overzealous methods can kill up to 70% [22]. In addition, particular patient populations might need special attention regarding the homogenization and decontamination method to be used. This is especially true for respiratory specimens from patients with cystic fibrosis because it has been reported that NTM are being recovered from these patients with increasing frequency. About 80% of these specimens, however, also contain *Pseudomonas aeruginosa*, which can overgrow the culture medium and thus prevent the isolation of NTM. Recently, it was demonstrated that the N-acetyl-L-cysteine/NaOH decontamination method, followed by a 5% oxalic acid treatment, can sufficiently reduce the overgrowth by *P. aeruginosa*, thus improving the recovery rate of clinically significant NTM [22,47].

The Centers for Disease Control and Prevention (CDC) recommend the use of both liquid and solid media in order to decrease the time to detection and to increase the yield of growth detection [33]. For many years, the only culture system with the potential to decrease turnaround time was the BACTEC 460TB system (Becton-Dickinson Diagnostic Instrument Systems) [48]. It has been shown, however, that the newly introduced Mycobacteria Growth Indicator Tube (MGIT; Becton-Dickinson Diagnostic Instrument Systems), BACTEC 9000 MB (Becton-Dickinson Diagnostic Instrument Systems), MB Redox (Biotest AG, Dreieich, Germany), MB/BacT (Bio Mérieux, Inc, Durham, NC) and ESPII (Accumed, Inc, Westlake, Ohio) systems are suitable nonradio metric and/or fully automated alternatives to the radiometric BACTEC 460 TB system [49–52]. It is noteworthy that, although all the alternative broth-based systems showed a comparable sensitivity to the BACTEC 460 TB system, the MGIT system exhibited a significantly better recovery rate regarding the *M. avium* complex and other NTM (86% versus 72%, and 69% versus 50%, respectively), and the ESPII showed a higher recovery rate regarding the *M. avium* complex (94.6% versus 75.7%) [50,53]. But with the exception of the BACTEC 460 TB and the BACTEC 9000 MB systems, the novel growth detection systems cannot be used for direct inoculation of blood. Blood samples can be inoculated into these systems only after lysis and centrifugation steps [49,50,53–55].

Although the broth-based systems have decreased the time to detection to 1–3 weeks, a solid medium should be used for those strains that may not grow well in liquid media [48–51]. In particular, this holds true for *M. haemophilum*, which will grow better on solid media (supplemented with hemin or hemo globin as an iron source) [56]. Therefore, all acid-fast microscopy positive specimens, specimens from ulcerative skin lesions or septic joints of immuno-compromised patients, specimens from undiagnosed pulmonary lesions of bone marrow transplant recipients, and specimens from children with adenitis should also be inoculated either onto chocolate plates, or to Middlebrook 7H10 agar with hemolyzed sheep erythrocytes, hemin, or an X-factor strip, or to a Lowenstein-Jensen (LJ) slant with 1% ferric ammonium citrate [19,56]. Broth media also can be supplemented with X-factor strips [19]. It is noteworthy that *M. avium* subspecies *paratuberculosis* also requires additional nutrients (egg yolk and the siderophore mycobactin J) in both liquid and solid media for optimal growth [57].

Other species like *M. genavense*, however, show a better recovery rate in liquid media especially at an acidic pH (pH 5.5) [7]. Similar to liquid media, the pH of solid media can also significantly influence the growth of mycobacteria. It has been shown that for slowly growing mycobacteria (>7 days for visible growth in subculture), based on the testing of 16 different species, the optimal pH in LJ medium was between 5.8 and 6.5, with the exception of *M. leprae marium* (pH 5.8–6.1) [58]. As for rapid growers (visible growth of subculture in <7 days), the optimal pH was between 7 and 7.4, with the exception of *M. chelonae*, which preferred an acidic pH [58]. These findings indicate that the routinely applied LJ medium with pH 7 is not optimal for the isolation of all mycobacteria. Therefore, in areas endemic for lung or other diseases caused by NTM, inoculation to an additional LJ slant with an acidic pH, or to Ogawa medium (pH 6), is recommended [58].

A further advantage for culturing mycobacteria on solid media is that growth can be quantified, colony morphology and pigmentation can be examined, and biochemical tests can be performed if warranted. This additional information can also provide valuable clues to identify NTM or to direct the selection of other confirmatory tests such as DNA hybridization assays [59].

**Nucleic acid hybridization methods**

Use of the AccuProbe (Gen-Probe Inc, San Diego, CA) nucleic acid hybridization kits represented a quantum leap forward in the rapid identification of the MTB complex, the *M. avium* complex, *M. gordonae*, and *M. kansasi*. These assays allowed rapid identification of these mycobacteria (results within 2 hours) as soon as sufficient biomass is obtained following growth in culture [19]. In rare instances,
cross-reaction has been documented in the Accu-Probe for the MTB complex with isolates of either *M. celatum* types 1 and 3 or *M. terrae* when the test is not performed precisely as indicated in the package insert [60–62]. Adherence to the proper hybridization temperature (between 60°C and 61°C rather than 60°C ± 1°C) was the most critical parameter [62].

Although *M. gordonae* has not been associated with disease, it is one of the most commonly isolated mycobacterial species. Therefore, rapid identification of this organism using a DNA probe has facilitated patient management by ruling out involvement of mycobacterial pathogens. A recent study found, however, that not all strains of *M. gordonae* were positive with this species-specific probe [63].

As opposed to the nonpathogenic *M. gordonae*, *M. kansasii* is one of the major pulmonary mycobacterial pathogens. As previously stated, human-to-human transmission has not been established for *M. kansasii* and other NTM, with the environment, particularly tap water, being the most likely source. In the past, identification of *M. kansasii* in the clinical laboratory has relied on growth characteristics, pigmentation, and selected biochemical tests. In recent years, identification of *M. kansasii* has been greatly facilitated by use of the commercially available DNA probe (AccuProbe, Gen-Probe, Inc); however, it has been shown that not all subspecies of *M. kansasii* are positive with this probe [64]. In order to solve this problem and identify all variants, a new version of the *M. kansasii* AccuProbe was developed and made available [65]. In a study published in 1999, this newer version was found to identify 97.4% (113/116) of the *M. kansasii* strains tested [66]. These results demonstrate that, although rare, there is still a possibility that some strains of *M. kansasii* may be falsely negative with the commercial probe. This caveat should be considered when other assays lead to the identification of an isolate as *M. kansasii*.

Because DNA/RNA probe assays do not include an amplification step, these tests are not sensitive enough to be used directly on clinical specimens; however, DNA/RNA probes are usually capable of identifying mycobacteria in contaminated liquid cultures depending on the extent of the contamination [67] because they have a sensitivity and specificity of nearly 100% when at least 10^5 organisms are present [68]. Unfortunately, commercial probe assays are not available for the majority of the NTM.

**High-performance liquid chromatography**

High-performance liquid chromatography (HPLC) of mycolic acids of *Mycobacterium* species has proved to be a rapid and reproducible tool to identify a wide range of known or unknown mycobacteria species either from culture or from sputum [69–71]. Standardized procedures and pattern standards have been published by the HPLC Users Group and CDC (www.cdc.gov/ncidod/dasitl/TB/TB_HPLC.htm) [71]. Species identification by mycolic acids is made by comparison with in-house databases. But a computer-based pattern recognition method and a site on the World Wide Web (http://hplc.cjb.net), maintained by the HPLC Users Group for specific support of the HPLC identification of mycobacteria, are also available [71,72].

The most commonly used ultraviolet (UV) HPLC is not as sensitive as the fluorescent HPLC, an adaptation that can significantly increase the sensitivity of HPLC while reducing the cell mass and the time required for mycobacterial identification. Although not as sensitive as NAA assays, fluorescent HPLC is being used on a limited basis for rapid direct testing on sputum [69]. Overall, HPLC is rapid (<2 hours) and the cost of consumables are inexpensive; however, the assay requires a dedicated and highly trained technician (because of the visual interpretation of the chromatographic patterns), costly instruments and software, expertise on instrumentation maintenance, and standardized growth conditions including the need for a large biomass when using UV-HPLC [71].

**PCR and restriction fragment length polymorphism analysis**

The concept of differentiating among slowly growing mycobacteria by PCR and restriction fragment length polymorphism analysis (PRA) using the *hsp65* gene was developed by Plikaytis et al in 1992 [73]. Similar assays targeting the 16S rRNA or the *dnaJ* genes were subsequently evaluated, but ultimately a method based on the *hsp65* gene was developed for use in routine clinical practice [74–76]. This rapid test can be done on AFB isolates that grow either in liquid or on solid media. Because of the amplification by PCR, the assay requires less biomass than either the AccuProbe or HPLC. Originally, Telenti et al identified 33 PRA patterns of which 19 corresponded to a single species, and 14 were associated with 5 species [74]. Later, Taylor et al added 5 additional patterns (1 more species and 4 new subtypes), and Devallois et al introduced another 11 patterns (5 additional species and 6 subtypes) into this algorithm [77,78]. A website that contains band patterns for reference for those using the PRA procedure is also available (www.hospvd.ch/prasite).
Recently, Brunello et al. developed a modified PRA of the hsp65 gene with a new algorithm describing 54 species, including 22 species not described previously [79]. In this assay, restriction digests were separated by 10% polyacrylamide gel electrophoresis (PAGE) instead of agarose gel electrophoresis. They found that 10% PAGE analysis provided a more precise estimate of the size of the restriction fragments and allowed the identification of mycobacteria whose PRA patterns contained fragments shorter than 60 bp.

A drawback of PRA is misidentification because of intraspecies genetic variability (if the PRA pattern is not distinct). Intraspecies variability can be used, however, as molecular epidemiologic markers for particular NTM if the PRA pattern is distinct. In order to overcome this problem, two new diagnostic algorithms were developed based on the PRA of the 16S-23S DNA spacer region and on the PRA of the rpoB gene of mycobacteria [80,81]. The first method identified 48 species, 40 subspecies, and 4 subtypes, whereas the second method identified 50 species and 13 subtypes. Both methods were proposed as an alternative to hsp65 PRA.

The commercially available (except in the United States) kit-based Inno-LiPA Mycobacteria assay (Innogenetics N.V., Ghent, Belgium) also targets the 16S-23S DNA spacer region for identification of mycobacteria [82–84]. The LiPA method is based on the solid-phase reverse hybridization of biotinylated PCR amplicons of the target region to oligonucleotide probes arranged on a membrane strip. The system is capable for the detection and identification of the MTB complex, the M. avium complex, M. kansasii, M. xenopi, M. gordonae, M. scrofulaceum, and M. chelonae from solid and liquid media. A major advantage of this method is the possibility of the simultaneous detection of species in mixed cultures [82].

DNA sequencing

DNA sequencing of variable genomic regions offers a more rapid and accurate identification of mycobacteria compared with conventional phenotypic methods. In addition, it is also capable of providing phylogenic information about the relatedness of strains [4]. DNA sequencing methods are based on the determination of species-specific nucleotide sequences, which for identification are then compared to known sequences of in-house or commercially available databases [85]. The most routinely used and reliable method of this kind is the amplification and sequence analysis of hypervariable regions of the gene encoding 16S rRNA [86–88]. Using automated sequencers, the assay can be completed and identification results can be reported within 1–3 days. The installation, maintenance and running of automated DNA sequencing on a daily basis are expensive and laborious tasks, however.

Nevertheless, DNA sequencing has enabled the discovery of new NTM species that can be non- or poorly cultivable, potentially pathogenic, or not yet characterized [4,19]. It has also shown that the Mycobacterium genus is more diverse than it was assumed on the basis of the less-accurate conventional identification methods [89]. As a note of caution, a recent study demonstrated that clear-cut results with the 16S rRNA gene sequencing are not the rule because public or commercial databases may be inaccurate or may not include all established species [90]. One proposed solution is the Ribosomal Differentiation of Medical Microorganisms system (RIDOM), which is a quality-controlled service, freely available on the Internet for mycobacterial identification by 16S rRNA analysis [90]. Besides sequence data, RIDOM also contains additional information on clinical, phenotypic, and genotypic characteristics of the established and yet to be established mycobacterial strains.

Alternative DNA sequencing methods have been described for the characterization of mycobacteria. These assays are based on the amplification of the rpoB, gyrB, hsp65, and 32-kDa protein genes or the 16S-23S rRNA gene spacer [91–95].

DNA chip technology

A rapidly developing technology that appears promising for clinical mycobacteriology laboratories involves oligonucleotide arrays or “DNA chips” where molecular biology meets computer technology [96]. Gingeras et al [97], using an array designed to determine the specific nucleotide sequence diversity in 10 species of mycobacteria, examined 121 mycobacterial isolates using both DNA sequencing of the rpoB and 16S rRNA genes and analysis of the rpoB oligonucleotide array hybridization patterns. Species identification for each of the isolates was similar, irrespective of the method used.

The potential of the DNA chip technology was also demonstrated by another recent study by Troesch et al [98]. As in the previous study, the use of a DNA probe array was based on two sequence databases. The first served species identification (82 unique 16S rRNA sequence patterns from 54 species) and the second, detection of rifampin resistance in MTB (rpoB alleles). Seventy mycobacterial isolates from
27 species and 15 rifampin-resistant MTB strains were tested. The same hybridization conditions could be used for both genes and the platform that was described can be expanded to analyze other genes simultaneously as well. Epidemiologic markers could also be added to the array for tracing transmission links between strains. With the potential to perform direct testing of clinical specimens for identification and drug susceptibility and genotyping in one step, the DNA chip method hopefully represents the near future of the clinical mycobacteriology laboratory.

**Genotyping**

Recent technical advances in the field of molecular biology, in conjunction with an increased understanding of the molecular genetics of mycobacteria, have provided the means to type either MTB or NTM reliably at the DNA level [99]. Genotyping or DNA fingerprinting of NTM provides data to assess (1) strain relatedness, (2) the epidemiology of NTM infections and diseases, and (3) whether a positive culture result was caused by cross-contamination in the laboratory [4,19,45,46]. Restriction fragment length polymorphism (RFLP) assays targeting the insertion elements IS1245, IS1311, and IS901 have been developed to type and track specific *M avium* complex strains [100–103]. A standardized and computer-assisted protocol was also proposed for this species [104,105]. Molecular characterization methods for other NTM like *M kansasii*, *M xenopi*, *M celatum*, *M chelonae*, *M abscessus*, and *M malmoense* based on DNA fingerprinting or randomly amplified polymorphic DNA-PCR genotyping have also been useful tools in the recognition and control of laboratory contamination, nosocomial outbreaks, and pseudo-outbreaks [64,106–111].

**Susceptibility testing**

In contrast to tubercle bacilli, susceptibility testing of NTM awaits standardization. Only a few general guidelines for specific susceptibility testing are available, including the testing of isolates of clinically significant rapid growers (*M fortuitum*, *M chelonae*, and *M abscessus*), and the slow-growing *M kansasii*, and *M avium* complex. At present, there is no sufficient amount of experience with other NTM to recommend a standard susceptibility testing method for these species [3,112].

Susceptibility testing of the *M avium* complex for the first-line antituberculosis drugs is not recommended [3,112]. But in the following circumstances, *M avium* complex isolates should be tested against clarithromycin: (1) clinically significant isolates from patients on prior macrolide therapy, (2) isolates from patients with disseminated disease receiving macrolide preventive therapy, and (3) isolates from patients who have failed prior macrolide therapy or prophylaxis. Isolates of patients on azithromycin therapy should be characterized on the basis of clarithromycin susceptibility values as testing for azithromycin is problematic because of poor solubility [3,112].

*M kansasii* infections are usually treated with rifampin, isoniazid, and ethambutol. As discrepancies between in vitro susceptibility testing results for isoniazid and ethambutol and the clinical outcome are common, routine testing of these drugs are not recommended [3,112]. All initial clinically significant isolates of *M kansasii* should be tested against rifampin, however, by either the agar proportion method or BACTEC 460 TB [3,112]. Rifampin susceptibility testing should also be performed when the smear and/or culture remain positive after 3 months or when a relapse occurs during adequate therapy. In case of rifampin resistance, the isolate can be tested against rifabutin, isoniazid, ethambutol, streptomycin, amikacin, clarithromycin, ciprofloxacin, and sulfamethoxazole [3,112]. Susceptibility testing should be performed on all clinically significant isolates of rapidly growing mycobacteria [3,112]. The testing of isolates of patients with treatment failure or relapse is also necessary [3,112]. It is recommended that isolates should be identified at the species level, or at least the *M fortuitum* complex should be differentiated from *M abscessus* and *M chelonae* [3,112]. The testing of the following drugs are recommended as a primary panel: amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, imipenem, and sulfamethoxazole [3,112].

**Validation of new assays**

The newer assays described above have been developed as part of an ongoing quest for better products and new approaches. Any new method has to be validated on clinical samples, however. It is imperative that as many strains are tested for each NTM species as possible at several sites with a previously validated method. These strains should be well characterized. Acquiring the background information on the strains is also indispensable and will require close collaboration between laboratorians and clinicians. As only a few institutions have access to such well-characterized NTM strains, a repository of frozen strains should be formally organized and
aliquots made available to laboratories evaluating new assays. This approach would allow different procedures to be compared over time in different laboratories using the same group of well-characterized isolates. But besides using the isolates of these publicly available repositories, validation studies are also warranted on local strains from the laboratory that intends to implement a new method to avoid any misidentification due to intraspecies variability.

Assessing a laboratory’s performance

Quality control programs organized by the CDC and College of American Pathologists have revealed that there are significant differences between state and hospital based laboratories in the diagnostic methods available and, therefore, in the level of service and testing capabilities [113,114]. These findings indicate that to ensure high standards of mycobacterial testing all clinicians should be aware of the diagnostic methods, turnaround times for isolation, identification and susceptibility testing, and score of proficiency testing performed in their mycobacteriology laboratory.

Summary

In conclusion, it is important to realize that there is no “stand alone” assay for the identification of NTM. Many new species may not be recognized in all assays. Newer molecular tests are more accurate for identification than phenotypic tests and have significantly improved turnaround time. Clinical significance of an isolate should be determined, however, before committing resources for the identification of a mycobacterial isolate to the species level. In addition, there are significant differences in the range and quality of services provided by different laboratories. Today, techniques and equipment are increasingly complex and costly, making it more difficult to upgrade every local laboratory to perform these assays. But because specimen delivery and communication of results can be rapidly and easily achieved, utilization of reference laboratories for rarely performed sophisticated tests is a more practical approach.

References


Butler WR, Guthertz LS. Mycolic acid analysis by high-performance liquid chromatography for identifi-


