

Laboratory Diagnosis of Mycobacterial Infections: New Tools and Lessons Learned

Yvonne M. Hale,¹ Gaby E. Pfyffer,² and Max Salfinger³

¹Bureau of Laboratories, Florida Department of Health, Jacksonville, Florida; ²Swiss National Reference Center for Mycobacteria, Department of Medical Microbiology, University of Zurich; and ³Wadsworth Center, New York State Department of Health, Department of Medicine, Albany Medical College, and Department of Biomedical Sciences, School of Public Health, State University of New York at Albany, Albany, New York

Even in the 21st century, tuberculosis continues to be a problem. Although the number of cases continues gradually to decrease in the United States, cases get more difficult to treat, specifically those that are multiple-drug resistant. Infection of one-third of the world's population ensures that tuberculosis will not disappear in the near future. In light of this, it will be useful to know the goals for the health care system and how these goals may be accomplished. Laboratory testing in the mycobacteriology field is experiencing more changes today than ever before. Determining what assays will be most useful to the clinician is a challenge, and acceptance of the new technology by the medical community an even greater one. Clinicians must use the best available resources to determine the most appropriate care for their patients and work together with the laboratory to ensure that the communication channels are open. This review focuses on current state-of-the-art resources useful for accurate and rapid laboratory diagnosis of mycobacterial infections.

Despite the progress in promoting the public's health and ensuring quality care, communities in the United States have been surprised by the resurgence of an old disease with a new twist: drug-resistant tuberculosis. How could this happen? As the incidence of tuberculosis declined in the 1960s and early 1970s, so did programs to control it [1]. Beginning in 1980, the seeds for an impending disaster were sown. Federal funding targeted specifically for tuberculosis control was phased out and replaced with general public health block grants to the states. Many states and cities decided to spend less money to fight tuberculosis. It has been >15 years since the Centers for Disease Control and Prevention (CDC) first observed a deviation from the expected decline in tuberculosis [2]. Although the resurgence of tuberculosis in inner cities has been largely attributed to the HIV epidemic, Brudney and Dobkin [3] clearly showed that worsening economic and social conditions, including an increase in homelessness, have contributed substantially to an increase in tuberculosis. Since 1993, the incidence of tuberculosis has been

declining again. However, the cost to contain the resurgence of tuberculosis has been phenomenal. In New York City, for example, there were >20,000 excess cases of the disease from 1979 through 1994 that would not have occurred if previous downward trends had continued. The resulting costs were estimated to easily exceed \$1 billion [1]. The challenge in the years to come will be to increase the political will to fight tuberculosis, and thus eliminate it not only in New York City but in the entire United States and beyond.

The emergence of strains of *Mycobacterium tuberculosis* that are resistant to antituberculosis agents, although not a novel phenomenon, has recently received increased attention, largely because of the dramatic outbreaks of multidrug-resistant tuberculosis in HIV-infected patients in New York and Florida [4]. Delayed diagnoses, inadequate treatment regimens, high mortality, and significant rates of nosocomial transmission have characterized these outbreaks [5].

Overall, the World Health Organization estimated that there were >8 million cases of tuberculosis in 1998, 80% concentrated in 22 high-burden countries, led by India and China [6]. Drug-resistant tuberculosis became a worldwide problem in both immunocompetent and HIV-infected populations [7, 8]. Although progress is noticeable, control of tuberculosis is hampered by the fact that tuberculosis and HIV disease are closely linked in a mutually disadvantageous synergy: HIV infection

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Reprints or correspondence: Dr. Max Salfinger, Wadsworth Center, New York State Department of Health, Albany, NY 12201-0509 (salfinger@wadsworth.org).

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promotes the progression of *M. tuberculosis* infection to disease, and tuberculosis accelerates the course of HIV disease [9].

In 1975, the genus *Mycobacterium* comprised some 30 species; 25 years later, it comprises close to 100. The newly discovered nontuberculous mycobacterial species described since 1990 and their origin, whether from clinical specimens or elsewhere, are listed in table 1. This plethora of species poses an additional challenge for the clinical mycobacteriology laboratory to provide timely services. Furthermore, some of the more recently described species (e.g., *Mycobacterium haemophilum*, *Mycobacterium genavense*) require special growth conditions, necessitating an exquisite collaboration between the clinician requesting the test and the laboratory professional performing the test.

M. tuberculosis, *Mycobacterium bovis*, and other entities within this taxon (*M. bovis* bacille Calmette-Guérin [BCG], *Mycobacterium africanum*, *Mycobacterium microti*, and *Mycobacterium canettii*) are referred to as the *M. tuberculosis* complex, and many clinical microbiology laboratories identify these organisms only to the level of the complex. This practice is supported by commercial kits, such as the AccuProbe assay (Gen-Probe); nucleic acid amplification-based (NAA) techniques; and the radiometric NAP (*p*-nitro- α -acetylaminobeta-hydroxypropionophenone) assay, none of which can differentiate between members of the *M. tuberculosis* complex. Such incomplete identification can hamper patient management, in the case of BCG dissemination as a complication of BCG immunostimulation against bladder cancer [40], and can undermine

Table 1. Newly discovered nontuberculous mycobacterial species described since 1990.

Year described	<i>Mycobacterium</i> species	Reference (first description)	Rate of growth	Isolated from clinical specimens
1990	<i>M. cookii</i>	[10]	Slow	No
1992	<i>M. abscessus</i>	[11]	Rapid	Yes
1992	<i>M. alvei</i>	[12]	Rapid	Yes
1992	<i>M. confluentis</i>	[13]	Rapid	Yes
1992	<i>M. peregrinum</i>	[11]	Rapid	Yes
1993	<i>M. brumae</i>	[14]	Rapid	Yes
1993	<i>M. celatum</i>	[15]	Slow	Yes
1993	<i>M. genavense</i>	[16]	Slow	Yes
1993	<i>M. hiberniae</i>	[17]	Slow	No
1993	<i>M. intermedium</i>	[18]	Slow	Yes
1993	<i>M. interjectum</i>	[19]	Slow	Yes
1994	<i>M. chlorophenicum</i>	[20]	Rapid	No
1995	<i>M. branderi</i>	[21]	Slow	Yes
1995	<i>M. conspicuum</i>	[22]	Slow	Yes
1995	<i>M. mucogenicum</i>	[23]	Rapid	Yes
1996	<i>M. hodleri</i>	[24]	Rapid	No
1996	<i>M. lentiflavum</i>	[25]	Slow	Yes
1996	<i>M. triplex</i>	[26]	Slow	Yes
1997	<i>M. hassiacum</i>	[27]	Rapid	Yes
1997	<i>M. heidelbergense</i>	[28]	Slow	Yes
1997	<i>M. mageritense</i>	[29]	Rapid	Yes
1997	<i>M. novocastrense</i>	[30]	Rapid	Yes
1998	<i>M. bohemicum</i>	[31]	Slow	Yes
1999	<i>M. goodii</i>	[32]	Rapid	Yes
1999	<i>M. murale</i>	[33]	Rapid	No
1999	<i>M. tusciae</i>	[34]	Slow	Yes
1999	<i>M. wolinskyi</i>	[32]	Rapid	Yes
2000	<i>M. botniense</i>	[35]	Slow	No
2000	<i>M. septicum</i>	[36]	Rapid	Yes
2000	<i>M. kubicae</i>	[37]	Slow	Yes
2000	<i>M. elephantis</i>	[38]	Rapid	No
2000	<i>M. heckeshornense</i>	[39]	Slow	Yes

important public health investigations, in the case of possible cattle-to-human transmission of *M. bovis* [41]. In addition, *M. microti* [42], *M. africanum*, and *M. canettii* [43] should be recognized at the species level to enable collection of more information about their epidemiology.

Thus, the mycobacteriology laboratory plays an important role in primary care and public health by providing more accurate results and having a shorter turnaround time than do less-specialized clinical laboratories. The laboratory diagnosis of mycobacterial infections, tuberculosis in particular, must be expedited to improve better patient management and to save limited resources within the health care system. In 1986, the British Medical Research Council published data about the influence of initial drug resistance on the response of patients with pulmonary tuberculosis to short-course chemotherapy [44]. In contrast to the high success rate of short-course regimens in cases with initial resistance to isoniazid and streptomycin, the response of the few cases with initial resistance to rifampin was poor. Therefore, it is imperative to recognize rifampin resistance as early as possible, to allow for adequate adjustment of the drug-treatment regimen. The 2 most urgent questions in a suspected case of tuberculosis, which need to be rapidly addressed by the mycobacteriology laboratory, are

whether there are tubercle bacilli involved and, if so, whether they are resistant to rifampin. A model for quick and accurate laboratory services is that offered by the FAST TRACK program in Florida, whose services are summarized in table 2.

The mycobacteriology laboratory has passed through several phases in its effort to answer these questions accurately and more rapidly: from using radiometric growth detection, including susceptibility testing, in the mid-1980s, to using nucleic-acid probes in the late 1980s, NAA in the mid-1990s, and now DNA sequencing, which is available in a kit format. This review updates recent developments in laboratory diagnostics and provides ideas about how clinicians can assess and access laboratory services. In addition, the review details the responsibilities carried out by public health bodies and professional societies to ensure access to newer technologies and described realistic goals that may help to drive the future of mycobacteriology laboratory testing.

THE CLINICIAN-LABORATORY RELATIONSHIP

In recent years, there has been a growing body of new and exciting methods in mycobacteriology, but there is not yet any single test for tuberculosis that can stand alone [45]. Most

Table 2. Services offered by the Florida Department of Health's FAST TRACK Program.

Service	Times available	Comments
Staffing	Daily	—
Microscopy	Daily	24-h turnaround time from receipt of specimen to availability of AFB smear results, which are performed with use of fluorochrome staining and confirmed with use of carbol fuchsin staining; first-time positive results are reported to the health care provider by telephone
Nucleic acid amplification	Monday–Saturday	Used to determine rapidly (in ≤ 1 day) whether a smear-positive sputum sample contains <i>Mycobacterium tuberculosis</i> complex; available for smear-negative specimens only on request
Detection of growth in culture	Daily	Up to 6 weeks time to detection with use of BACTEC 12B liquid medium, Löwenstein-Jensen slants (glass test tubes; egg-based medium), 7H10 or selective 7H11 biplates (agar-based medium); report issued as soon as growth of mycobacteria has been detected
PCR restriction enzyme analysis	Daily	Primary means to rapidly identify and confirm <i>M. tuberculosis</i> complex or nontuberculous mycobacteria in culture
DNA probes	Daily	Used to rapidly identify and confirm <i>M. tuberculosis</i> complex or <i>M. avium</i> complex in culture
HPLC	Weekly	Used to identify nontuberculous mycobacteria
Susceptibility testing	Daily	Determines BACTEC 12B liquid-media susceptibilities for first-line and second-line antituberculosis drugs; drug resistance confirmed by proportion method
DNA typing	On request	Performed only for <i>M. tuberculosis</i> complex strains

NOTE. AFB, acid-fast bacilli.

important, complementary techniques should be used to generate complete and rapid information. The laboratory director needs to decide which tests will be best performed in-house and which specimens should be sent to a reference laboratory, on the basis of the community to be served and the resources available and in consultation with the infectious disease specialists, pneumologists, an/or other physicians involved. When this partnership is established, the physicians will 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 the clinician should notify the laboratory when results are not consistent. An established and ongoing professional relationship between clinicians and the laboratory enables the recognition of inaccurate results earlier and, therefore, may minimize the potential harm to the patient. Previous reports [46, 47] have demonstrated that ~3%–4% of cultures for tuberculosis have false-positive results. In a study that analyzed drug-resistant tuberculosis [48], 13% of cultures had erroneous results; furthermore, in a study of specimens from patients with negative results of smears for acid-fast bacilli (AFB) and only 1 positive culture result [49], erroneous results were reported for 56% of cultures. However, it is possible that the error may have occurred in the pre-testing phase, such as by use of a nonsterile bronchoscope for specimen collection [50] or by detection of residual amplifiable *M. tuberculosis* DNA that could have remained in the sterile bronchoscope [51]. These reports underscore the facts that laboratory results alone (i.e., positive culture result or drug resistance) are not enough to dictate a particular strategy for the patient's care and that a careful clinical correlation is necessary to make 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.

QUALITY TESTING REQUIRES A QUALITY SPECIMEN

Accurate, rapid microbiological diagnosis of tuberculosis and other mycobacterial infections begins with proper specimen collection and rapid transport of the specimen to the laboratory. To ensure collection of the best possible specimen, the health care worker has to be properly trained, and the patient has to be provided with clearly presented instructions that they fully understand. The clearly labeled specimen must be transported to the laboratory quickly, because results of some tests, such as AFB smears and NAA procedures, can reliably be reported within 24–48 h of receipt in the laboratory.

To provide the best results, the volume of a sputum specimen should exceed 5 mL [52]. In addition, other specimens may

be collected, such as urine, CSF, pleural fluid, bronchial-wash samples, blood (reserved for immunocompromised patients, especially patients with AIDS), material from abscesses, endometrial scrapings, bone marrow, and other biopsy specimens [53, 54]. Aerosol-producing procedures should be done in a way that ensures the safety of the health care worker during collection [55].

Most specimens will contain microorganisms other than mycobacteria. Therefore, the specimen has to be refrigerated if a delay should occur, because otherwise overgrowth of the more rapidly growing contaminants may occur. Unfortunately, very often the laboratory is at the end of the decision tree for the patient's health improvement, especially in situations in which the patient waits weeks until seeing a physician, the mycobacterial disease is not initially recognized, or the specimen submitted to the laboratory is substandard. The importance of providing a high-quality and timely specimen should be conveyed to the health care provider. Health care providers and laboratorians must communicate to ensure the best laboratory testing.

REGULATIONS FOR PACKAGING AND SHIPPING LABORATORY SPECIMENS

Laboratories must maintain the integrity of patient specimens before the specimens are analyzed. Maintaining specimen integrity in the laboratory is not simple, and it becomes even more complex when specimens are transported by public conveyance. Specimens must be kept from leaking and from being crushed in unexpected accidents. They must be packed to protect both the specimens and those who handle them [56].

The regulations that apply depend on how the specimen is sent. Each mode of transportation has its own rules. For specimens sent by mail, the US Postal Service regulations regarding dangerous goods should be followed. For specimens sent by ground courier, Department of Transportation regulations should be followed: regulations governing specimen transport are incorporated in a document referred to as "49 CFR" [57]. If specimens are sent by air, the dangerous goods regulations of the International Air Transportation Association and International Civil Aviation Organization must be followed.

SAFETY

Safety in the mycobacteriology laboratory is of paramount concern. Sputum samples and other clinical specimens from patients with known or suspected tuberculosis must be considered potentially infectious. Aerosols must be controlled by the use of biological safety cabinets and centrifuges with safety carriers, and the laboratory staff must follow safety guidelines. All work involving specimens or cultures must be performed in a bio-

logical safety cabinet. This includes making smears, inoculating media, adding reagents, opening centrifuge cups, sonication, and any other potentially aerosol-producing procedures. The use of respirators may be warranted for work in a biosafety level 3 (BSL-3) laboratory and in a laboratory that grows large amounts of *M. tuberculosis*, works with drug-resistant isolates, or performs tasks with unknown risks. BSL-3 practices require restricted laboratory access, directional air-flow with negative pressure, and the wearing of special laboratory clothing and gloves. Respirators should be certified by the National Institute for Occupational Safety and Health. A minimal-level respirator is the N-95 respirator; a purified air-powered respirator is an option that some laboratories have found to be more comfortable for technicians working many hours at a biological safety cabinet. A respirator program should be implemented when respirator use is necessary. Personnel should be regularly monitored by performing a tuberculin skin test at least annually [54, 58].

TESTING AND PRIORITIZING SPECIMENS

Regardless of the many recent advances in implementing molecular techniques in the mycobacteriology laboratory, microscopic examination for AFB remains an inexpensive and rapid means for identifying highly infectious tuberculosis patients. It allows a quantitative estimation of the number of bacilli being excreted and therefore remains the cornerstone of infection control. Smear results should be available for the physician within 24 h of specimen collection [55] or, if an off-site laboratory is used, within 24 h after they receive the specimen [59]. Whereas the specificity of microscopic examination for AFB is excellent (89%–100%), microscopic examination has 2 serious drawbacks: it is unable to distinguish tuberculosis bacilli from nontuberculous mycobacteria, and it has low sensitivity [60, 61]. High-volume laboratories may use fluorescent staining to reduce the time for screening slides and to increase sensitivity.

Molecular tests, such as NAA assays, should be used for rapid confirmation of suspected tuberculosis cases in patients who have a sputum specimen positive by AFB smear [62]. Since 1999, the *Mycobacterium tuberculosis* direct test (MTD; Gen-Probe) has also been approved for AFB smear-negative respiratory specimens. NAA tests, including PCR, transcription-mediated amplification, ligase chain reaction, and strand displacement amplification, may enhance diagnostic certainty, particularly for patients for whom prompt treatment is imperative. However, the results of molecular methods, like other laboratory findings, should always be interpreted in conjunction with the patients' clinical data [63]. Pfyffer et al. [64] showed that NAA techniques had similar sensitivity and specificity overall for respiratory and nonrespiratory specimens. These tests can be done in as few as 3 h, allowing same-day

reporting of results. These tests should be done only in laboratories that are proficient in their use. However, an NAA method does not replace the need to perform cultures, because culture is still required for the identification of nontuberculous mycobacteria, as well as for susceptibility testing of a specimen that contains *M. tuberculosis* complex.

Few studies have addressed the use of NAA assays for non-respiratory specimens [64, 65]. Paradoxically, for extrapulmonary tuberculosis (e.g., tuberculosis meningitis), a rapid and accurate laboratory diagnosis is of prime importance because, often, a smear has negative results and cultures yield *M. tuberculosis* only after several weeks, if at all. Tuberculosis of the CNS remains among the most malignant of all forms of human tuberculosis. It is responsible for high rates of death and neurological disability and is often very difficult to diagnose [66, 67]. Smears of CSF samples yield positive results for <10% of patients in some reports [68, 69]. Even though culture of CSF is also an unreliable diagnostic technique, a positive mycobacterial culture remains the reference standard for diagnosis of tuberculous meningitis [70, 71]. However, one may challenge the dogma that culture is the reference standard and instead stress that confirmed clinical diagnosis is the criterion against which new microbiological assays should be assessed. After they modified the pretreatment step for the MTD assay, Pfyffer et al. [64] reported a sensitivity of 93.1% and specificity of 97.0%. In another MTD study, the cutoff value was lowered from 30,000 relative lights units to 11,000, and sensitivity increased from 33% to 83%, while specificity remained 100% [72]. Bonington et al. [73] reported that the AMPLICOR test (Roche) had a sensitivity of 60% and specificity of 100% for detecting cases of definite and probable tuberculosis meningitis in patients treated for <10 days from whom CSF specimens had been obtained. AMPLICOR was more sensitive than the combination of AFB smear microscopy and radiometric growth detection.

Most recently, Caws et al. [74] reported data from a national molecular diagnostic service for tuberculosis meningitis in the United Kingdom. Their PCR method, targeted to the genetic element IS6110, revealed a sensitivity of 75% compared with positive culture results and 35% compared with the final clinical diagnosis of tuberculosis meningitis. In contrast, culture yielded positive results in 17% of cases only when compared with clinical diagnosis. Of the diagnostic tests for tuberculosis meningitis that provide a better sensitivity than culture, NAA is currently the most rapid, although it still is not optimal. CSF specimens from patients suspected of having tuberculosis meningitis should be processed immediately. Lumbar punctures should be done with some forethought regarding the volumes of fluid and delivery speed needed for diagnosis. At least 5 mL of CSF should be submitted to the mycobacteriology laboratory as quickly as possible for culture and NAA testing.

In an international collaborative quality control study that involved 30 laboratories, Noordhoek et al. [75] assessed the reliability of NAA methods in detecting infection with *M. tuberculosis* complex. Each laboratory was asked to use their routine NAA technique to detect tuberculosis bacilli in a blinded panel of 20 sputum specimens containing 0, 100, or 1000 cells of *M. bovis* BCG. As reported in 1994 [76] in a preliminary study, results were, again, disappointing, inasmuch as only 16% of all laboratories were able to correctly identify the presence or absence of *M. bovis* BCG in the 20 samples. Irrespective of the targets and technology used, this study showed very elegantly that lack of specificity was more of a problem than was lack of sensitivity, that test reliability was not associated with any particular method, and, worst, that many of the participating laboratories did not use adequate quality control. These facts clearly underline the need for good laboratory practice and reference reagents to monitor the performance of NAA assays, including pretreatment of clinical samples.

Although the AFB smear cannot differentiate between *M. tuberculosis* complex organisms and nontuberculous mycobacteria, a molecular-biological approach that makes use of fluorescence in situ hybridization appears promising. Specific peptide nucleic-acid probes were used to detect *M. tuberculosis* complex organisms in positive broth cultures [77, 78] or in AFB-positive sputum smears [79]. This fluorescence in situ hybridization assay could be an asset in the armamentarium of peripheral laboratories that serve communities with a substantial fraction of nontuberculous mycobacteria diseases, because no amplification equipment is needed.

Before cultures for AFB can be performed, the majority of clinical specimens must be subjected to a pretreatment involving homogenization, decontamination, and concentration. This holds true for respiratory, gastric fluid, and urine specimens, as well as other specimens from nonsterile body sites, primarily to eradicate more-rapidly growing contaminants, such as normal flora bacteria and fungi, while not seriously affecting the viability of the mycobacteria. Specimens are cultured for the following reasons: culture is more sensitive than microscopy (it is able to detect as few as 10 bacteria/mL [80]); growth of the organisms is necessary for precise species identification; drug susceptibility testing requires viable culture of the organisms; and genotyping of cultured organisms can be used for epidemiological purposes or to detect false-positive results. The sensitivity of culture is 80%–85%, and its specificity is 98% [81, 82].

Use of liquid culture medium and at least 1 solid medium are recommended to increase the yield of mycobacteria [54]. For AFB smear-positive specimens, inoculation of an additional biplate that contains both a plain and a selective Middlebrook agar (e.g., 7H10 or selective 7H11 medium) enhances the quality of the test results. Broth-based systems have decreased the

time to detection to 1–3 weeks, compared with 3–8 weeks for growth on solid media [81]. However, a solid medium has to be used for those occasional strains that may not grow in liquid media. In particular, this holds true for *M. haemophilum*, which grows easily on a chocolate agar plate at 30°C. The decision to use a specific broth system depends on various factors: the labor-saving potential, the management of radioactive waste disposal, and the potential dangers associated with needle punctures. Growth on solid media should be quantified, whereas growth in liquid media cannot be similarly quantitated.

The genus *Mycobacterium* consists of almost 100 different species, all of which appear similar on AFB staining. Many of these can be isolated from humans, although many are found in the environment. A specialized laboratory should be able to provide a precise species identification of most AFB isolated from humans. In contrast, the distinction between pathogen and saprophyte is not always clear-cut for isolates from a given individual. Each mycobacterial isolate, like each patient, must be evaluated individually with respect to its potential to cause disease [83].

Use of nucleic-acid probe kits was a quantum leap forward in the rapid identification of *M. tuberculosis* complex, *Mycobacterium avium* complex, *M. avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, and *Mycobacterium goodii* in culture because results are available within 2 h [54]. In rare instances, cross-reaction has been documented when the test is not done precisely as indicated in the package insert [84–86]. The probe technology has a sensitivity and specificity of nearly 100% [87] when at least 10⁵ organisms are present, except for *M. kansasii* (87%) [88]. Thus, these probes are not sensitive enough to be used directly on sputum specimens, which should be tested by means of NAA. Also, differentiation by means of physiological, biochemical, and molecular tests should be done to identify the species within the *M. tuberculosis* complex, at least for *M. bovis*, *M. bovis* BCG, *M. microti*, *M. canettii*, and *M. africanum*.

Recently reported comparative genomic studies that make use of the complete DNA sequence of *M. tuberculosis* have provided information about regions of the genome that are deleted in most BCG derivatives and also in some other members of the *M. tuberculosis* complex [89]. Parsons et al. [90] have investigated the use of molecular amplification procedures to determine the presence or absence of specific regions of the genome in 88 well-characterized members of the *M. tuberculosis* complex. *M. bovis* BCG and *M. microti* were each found to have unique genotypes. Alternatively, *M. africanum* was found to be the most diverse genetically (with 5 different genotypes: 3 unique, 1 shared with *M. tuberculosis*, and 1 shared with *M. bovis*). On the basis of these results, a group of 5 assays has been proposed to rapidly differentiate between species within the *M. tuberculosis* complex [90].

Larger laboratories may use high-performance liquid chromatography (HPLC) for the analysis of mycolic acids (β -hydroxy- α -fatty acids) as a tool to identify mycobacteria from culture [91] and from sputum [92, 93]. Ultraviolet HPLC is not as sensitive as NAA assays done directly on sputum, but fluorescent HPLC is being used for rapid direct testing on sputum [93]. A distinct advantage of HPLC is the easy differentiation of *M. bovis* BCG from *M. tuberculosis* and *M. bovis* [94]. HPLC cannot, however, differentiate *M. bovis* from *M. tuberculosis*.

A PCR restriction-fragment-length polymorphism procedure based on the 65-kDa heat shock protein, which is capable of rapidly identifying many species of mycobacteria that are encountered in clinical practice, can also be used for the identification of AFB isolates that grow in liquid or solid media [95, 96]. This testing can be done with less biomass than required by either the AccuProbe or ultraviolet HPLC tests, and it can identify *M. tuberculosis* complex as well as many nontuberculous mycobacteria rapidly, ultimately leading to better patient management.

A still-developing technology that appears promising for clinical mycobacteriology laboratories involves oligonucleotide arrays or “DNA chips”—molecular biology meets computer technology. Gingeras et al. [97] used such an array, which has been designed to determine the specific nucleotide-sequence diversity in 10 species, to examine 121 mycobacterial isolates, both by means of conventional dideoxynucleotide sequencing of the *rpoB* and 16S ribosomal RNA genes and by means of analysis of the *rpoB* oligonucleotide array hybridization patterns. Species identification for each of the isolates was similar, irrespective of the method used.

Drug resistance is associated with large bacterial populations, as found, for example, in lung cavities caused by tuberculosis. Resistance that appears during drug treatment is due to selection and multiplication of naturally occurring mutants that are already present in the host's population of *M. tuberculosis*. Initially, antituberculosis drugs kill the susceptible organisms, which predominate in the population, and then the resistant organisms multiply. Killing of susceptible organisms and subsequent growth of drug-resistant mutants results in the “fall and rise” phenomenon demonstrated in sputum samples from patients in the late 1940s, which is described in a recent review of drug resistance in tuberculosis [98]. In 1986, the British Medical Research Council published data about the influence of initial drug resistance on the response to short-course chemotherapy for pulmonary tuberculosis [44]. They found that, in contrast to the high success rate for short-course regimens in patients whose infections were initially resistant to isoniazid and streptomycin, the response of the few patients with initial resistance to rifampin was poor. Because the recognition of

rifampin resistance is vital for the patient's outcome, susceptibility testing must be initiated as soon as possible.

Drug susceptibility tests should be done on initial isolates from all patients to decide on an effective antituberculosis regimen [53]. Additionally, drug susceptibility studies should be repeated if the patient continues to have culture results positive for mycobacteria after 3 months of treatment or if the disease reactivates. This approach has been found to be especially important during outbreaks of multiple-drug-resistant tuberculosis [53]. Drug-resistant tuberculosis should be suspected and the laboratory notified if affirmative answers are given to the following questions: Is the patient a contact of a known patient with drug-resistant tuberculosis? Has the patient been treated previously for tuberculosis? Is the patient having failure of tuberculosis treatment (i.e., are cultures still positive after 3 months)? Is the patient from a region or country with a significant incidence of drug-resistant tuberculosis?

It is well known that the radiometric BACTEC method (BD Biosciences) generates rapid results for the 5 first-line antituberculosis drugs (rifampin, isoniazid, ethambutol, streptomycin, and pyrazinamide) [99]. Because of the serious impact of resistant strains on patient management, drug resistance should be confirmed either by a second method or by a second laboratory—especially to rule out mixed cultures of *M. tuberculosis* and nontuberculous mycobacteria that will produce false resistance-patterns. In the case of drug resistance, second-line drugs may be tested by means of the proportion method [99, 100] or a radiometric protocol [101]. Susceptibility results should be reported without delay by telephone or fax to the health care provider, infection control, and the local tuberculosis control program [53].

Telenti et al. [102] demonstrated the usefulness of molecular techniques for detecting resistance by using PCR single-strand-conformation polymorphism analysis. Mutations in the *rpoB* gene (which encodes the β subunit of RNA polymerase) [103] were identified in all rifampin-resistant isolates and in none of the susceptible strains—although, due to operator error, the actual sensitivity of the blind study was 96%. More than 96% of rifampin resistance in *M. tuberculosis* correlates with mutations in an 81-bp segment of the *rpoB* gene. Not only does rifampin play a pivotal role in the treatment of tuberculosis, rifampin resistance is a surrogate marker for multiple-drug resistance in *M. tuberculosis* [104–106]. The now commercially available kit-based Inno-LiPA Rifotuberculosis assay (Innogenetics) performed equally well in identifying *rpoB* mutations [102] and can, furthermore, be used for the direct detection of *M. tuberculosis* complex at the same time. This technology has recently been extended to allow direct detection and identification of a number of nontuberculous mycobacteria, as well [107].

In contrast to the situation for tuberculosis bacilli, suscep-

tibility testing of nontuberculous mycobacteria awaits standardization. Only a few general guidelines for specific susceptibility testing are available, including the testing of isolates of clinically significant and rapidly growing species (including *Mycobacterium fortuitum*, *Mycobacterium chelonae*, and *Mycobacterium abscessus*), *M. kansasii*, and *M. avium* complex from patients who previously were treated with macrolides [83].

Recent technical advances in the field of molecular biology, in conjunction with a improved understanding of the molecular genetics of mycobacteria, have provided the means to type strains of *M. tuberculosis* reliably at the DNA level [108]. Genotyping or DNA fingerprinting of *M. tuberculosis* provides epidemiological data to assess whether a manifest tuberculosis episode is a reactivation of disease [109], a recent transmission of disease [110, 111], or an exogenous reinfection [112], or whether a positive culture result was due to cross-contamination in the laboratory or at bronchoscopy [113]. In addition, genotyping is able to give us an idea about how *M. tuberculosis* is transmitted in a community [114–116]. Most often, restriction-fragment-length polymorphism patterns are generated by targeting the genetic element IS6110, which is one of the numerous mobile genetic elements in mycobacteria. IS6110 is a naturally occurring transposable genetic element, which appears to be detectable in species of the *M. tuberculosis* complex only. Each strain of *M. tuberculosis* contains a different number of identical copies of this transposable element, except some Asian strains that are devoid of the IS6110 element [117–119]. The number of copies of IS6110 elements and the molecular size of the restriction fragments obtained after *PvuII* digestion vary in such a way that 2 unrelated strains do not produce identical patterns when hybridized with a labeled probe for IS6110, but produce unique genetic fingerprints.

A standardized protocol is available for strain identification by DNA fingerprinting [120]. Patterns and dendrograms, which illustrate the degree of relatedness among the isolates, can easily be assessed by computer analysis. Spoligotyping [121], as well as other methods, provide typing that is even more rapid, because as they use PCR to amplify the genetic material being typed. The CDC, through its National Tuberculosis Genotyping and Surveillance Network, offers rapid and accurate strain typing nationwide and at no cost. For submission of *M. tuberculosis* isolates to 1 of the 7 designated sites, the local state public health laboratory should be contacted.

LABORATORY SERVICES: WHAT SURVEYS TOLD US

In December 1991, the CDC, in collaboration with the Association of Public Health Laboratories, surveyed state and territorial mycobacteriology laboratories [122]. Twenty-seven percent of the laboratories (which process 31% of total specimens)

used the radiometric growth detection system, in addition to a solid medium. Seventy-one percent of the laboratories (which process 81% of total specimens) used nucleic-acid probes or HPLC for identification of mycobacteria. Finally, 20% of the laboratories (which process 80% of total specimens) were using the radiometric method for susceptibility testing of *M. tuberculosis*. The time required for reporting test results was variable. To identify *M. tuberculosis* in clinical specimens took an average of 31 days (range, 5–80 days) from the time of specimen receipt, and drug susceptibility results were available in 42 days (range, 18–76 days). Laboratories that used radiometric growth detection, nucleic-acid probes, or HPLC for identification and radiometric susceptibility testing reported susceptibility testing results in a mean of 31 days (SD, ± 11 days). No information was provided about the specimen load at the state and territorial public health laboratories compared with the load for the entire United States.

Because mycobacteriology laboratories play an important role in the control of tuberculosis, especially for drug-resistant cases, a questionnaire was sent in 1994 to 188 laboratories that hold a New York State permit in mycobacteriology to assess real turnaround times under field conditions for all respiratory specimens that were initially smear-positive [123]. There were records for 2620 patients and 3013 respiratory specimens that were considered to be initially smear-positive for mycobacteria. Culture results for these specimens were as follows: *M. tuberculosis* complex, 1554 specimens (52%); nontuberculous mycobacteria, 1160 specimens (39%); and negative culture result or contaminated, 334 specimens (11%). The time elapsed between the date that smear-positive sputum specimens containing *M. tuberculosis* complex were collected and the date that susceptibility testing results for rifampin were reported to the physician was 8–9 weeks for 80% of isolates. In contrast, the New York State Department of Health's Fast Track for Tuberculosis Testing program provided susceptibility testing results to clinicians in only 4 weeks for 80% of isolates.

These surveys were triggered by the resurgence of tuberculosis, the recognition of the synergism with HIV infection, and the need for rapid results from the mycobacteriology laboratory. They provided baseline data that revealed that newer assays were not widely in use in the United States. This can be contrasted to the list of the range of services offered by the Florida Department of Health's FAST TRACK program (table 2).

ASSESSING A LABORATORY'S PERFORMANCE

External quality control schemes. The College of American Pathologists, the largest provider of proficiency testing in this country, has offered a program in mycobacteriology since 1969 [124]. In recent years, the CDC's Public Health Practice Program Office has offered 2 different performance evaluation pro-

grams for the testing of *M. tuberculosis* complex: 1 for drug susceptibility testing, since 1994, and 1 for NAA testing, since 1997. As of September 2000, 130 US institutions were enrolled in the drug susceptibility testing program (which requires BSL-3 practices), and 100 were enrolled in the NAA testing program (which requires BSL-2 practices; personal communication, J.C. Ridderhof). The programs provide aggregate reports of testing results that are useful for self-assessment of performance. This information may allow individual laboratories to identify problems in laboratory testing protocols and/or testing algorithms. There are no enrollment fees for participation. More information is available at the CDC Web site, at <http://www.phppo.cdc.gov/mpep>.

Current Clinical Laboratory Improvement Amendments of 1988 regulations require ~40% of all federally registered test facilities to participate in a proficiency testing program approved by the Centers for Medicare and Medicaid Systems. The maintenance of clinical laboratory operating licenses at state and federal levels is chiefly dependent on successful performance on proficiency tests. However, whereas assessment for performance of compatibility testing and ABO blood group typing require a score of 100% to pass, and for performance of Papanicolaou smears, a score of 90%, the passing score for all other categories, including microbiology, is only 80% [125]. This sharply contrasts with the quest for fewer errors in the provision of health care [126–128].

Laboratory capabilities. How can the health care provider assess the performance of a laboratory and distinguish a state-of-the-art laboratory from a conventional one that has built-in testing delays? The following recommendations, given in the form of questions, may serve as a guide for the clinician who is attempting to assess laboratory methods; they cover standards of laboratory practice, turnaround times, and quality assurance [129].

1. Does the laboratory perform or have access to the newest technology, such as NAA tests, for testing of specimens from patients with AFB smear-positive sputum samples and for patients with clinical and radiological signs of tuberculosis in spite of AFB smear-negative sputum samples?
2. Does the laboratory inoculate specimens into at least 1 liquid and 1 solid medium for detection of growth of mycobacteria?
3. Does the laboratory use rapid identification tests for *M. tuberculosis* complex, such as nucleic-acid probe kits or HPLC?
4. Does the laboratory confirm the identification of drug-resistant strains of *M. tuberculosis* either by a second method or by a second laboratory?
5. Is the result of the AFB sputum smear available within 24 h of specimen collection?

In addition, the laboratory should be asked about its turn-

around times. Rapid reporting of results is paramount for correct patient management and essential for prompt evaluation of the contacts of the index case. Any case of tuberculosis must be reported to the public health authorities. The laboratory must make an effort to report results rapidly, including transmission of results by telephone, fax, and electronic information system. Turnaround time for susceptibility testing results is an excellent marker for overall laboratory performance. For example, for specimens that are initially smear-positive and culture-positive, the New York State Department of Health requires that, for *M. tuberculosis*, the laboratories monitor the time elapsed between the date the specimen is taken and the date the result of testing for susceptibility to rifampin is reported to the health care provider. In addition, the clinician may ask the tuberculosis laboratory about participation (and scores) in external quality control (proficiency testing) programs. If any of the answers are “no” or not satisfying (e.g., passing scores of only 80%–90%), the issue should be discussed with the laboratory director or another expert in the field. Alternatively, another laboratory may be chosen to ensure high standards of microbiological testing [9].

GOALS

In January 2000, the US Department of Health and Human Services published “Healthy People 2010—Understanding and Improving Health” [130], which lists the wide range of public health opportunities that exist in the first decade of the 21st century. The report discusses 467 objectives in 28 focus areas, and includes the following 4 tuberculosis targets: to reduce the incidence of tuberculosis, to increase the proportion of all tuberculosis patients who complete curative therapy within 12 months, to increase the proportion of contacts and other high-risk persons with latent tuberculosis who complete a course of treatment, and to reduce the average time it takes for a laboratory to confirm and report tuberculosis cases (see table 3).

The technology is already at hand: it is only a matter of organizing the most efficient laboratory service to meet the fourth target by 2010. With these targets in mind, care of the following patients should be expedited: patients with newly diagnosed AFB smear-positive sputum samples; patients with clinical and radiological signs of tuberculosis despite AFB smear-negative sputum samples; and patients who are suspects of having drug-resistant tuberculosis [131].

The Institute of Medicine report “Ending Neglect: The Elimination of Tuberculosis in the United States” [132] reviews the lessons learned from the neglect of tuberculosis between the late 1960s and the earlier 1990s and reaffirms a commitment to the goal of eliminating tuberculosis in the United States, which is defined as a case rate of <1 case per million population per year. The report states, “to meet this goal, aggressive and

Table 3. Goals of the "Healthy People 2010" initiative for tuberculosis [130].

Goal number and description	Target	Baseline value
14-11: Reduce tuberculosis	1 new case per 100,000 population per year	6.8 new cases per 100,000 population in 1998
14-12: Increase proportion of all tuberculosis patients who complete curative therapy within 12 months	90% of patients	74% of patients in 1996
14-13: Increase proportion of contacts and other high-risk persons with latent tuberculosis infection who complete course of treatment	85% of persons	62.2% of persons in 1997
14-14: Reduce average time for laboratory to confirm and report tuberculosis cases	2 days for 75% of cases	21 days for 75% of cases in 1996

decisive actions beyond what is now in effect will be required," and offers a quote from Goethe: "Knowing is not enough; we must apply; / Willing is not enough; we must do" [132]

We—health care providers, laboratorians, and public health practitioners—must strive for a new type of leadership that merges all of the different players involved into a synergistic network, making the whole of the virtual organization more effective than the sum of its parts.

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