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Practice Guidelines for Clinical Microbiology Laboratories: Mycobacteria

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This review is dedicated to Gerri S. Hall, who passed away in January 2016. She was a passionate leader in the field of clinical microbiology.

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SUMMARY Mycobacteria are the causative organisms for diseases such as tuberculosis (TB), leprosy, Buruli ulcer, and pulmonary nontuberculous mycobacterial disease, to name the most important ones. In 2015, globally, almost 10 million people developed TB, and almost half a million patients suffered from its multidrug-resistant form. In 2016, a total of 9,287 new TB cases were reported in the United States. In 2015, there were 174,608 new case of leprosy worldwide. India, Brazil, and Indonesia reported the most leprosy cases. In 2015, the World Health Organization reported 2,037 new cases of Buruli ulcer, with most cases being reported in Africa. Pulmonary nontuberculous mycobacterial disease is an emerging public health challenge. The U.S. National Institutes of Health reported an increase from 20 to 47 cases/100,000 persons (or 8.2% per year) of pulmonary nontuberculous mycobacterial disease among adults aged 65 years or older throughout the United States, with 181,037 national annual cases estimated in 2014. This review describes contemporary methods for the laboratory diagnosis of mycobacterial diseases. Furthermore, the review considers the ever-changing health care delivery system and stresses the laboratory's need to adjust and embrace molecular technologies to provide shorter turnaround times and a higher quality of care for the patients who we serve.

KEYWORDS BCG, mycobacterium, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, NTM, diagnostic algorithm, mycobacterial diseases, nontuberculous mycobacteria, review, tuberculosis

INTRODUCTION

Belonging to the only genus in the family *Mycobacteriaceae* are a diverse group of bacteria that differ widely in several traits, such as their pathogenic potential in humans and animals, reservoirs, and growth dynamics in culture. For the most part, mycobacteria can be divided into four major groups based on fundamental differences in epidemiology, association with disease, and the ability to grow *in vitro*: those that belong to the *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, and *Mycobacterium ulcerans* and those referred to as nontuberculous mycobacteria (NTM). This introductory section provides an overview of their taxonomy and representative species belonging to these four major groups involved in human infections.

Taxonomy of Mycobacteria

Mycobacterium spp. contain mycolic acids in their cell wall and share this characteristic with bacteria of other genera such as *Gordonia*, *Nocardia*, *Rhodococcus*, and *Tsukamurella*. This characteristic enables these bacteria to be differentiated from other bacteria based on staining techniques since the high mycolic acid content in the cell wall makes organisms resistant to decolorization with acid alcohol (i.e., "acid fast"). *Mycobacterium* spp. are aerobic, non-spore-forming, Gram-positive, acid-fast bacilli (AFB). They are nonmotile, and most of them are straight or slightly curved rods, with only a small number of species exhibiting some branching. Some species are pigmented yellow or orange when cultured, and this pigment may be constitutive (i.e., scotochromogenic) or induced only with exposure to light (i.e., photochromogenic), while other species never produce pigment (i.e., nonphotochromogenic). Compared to most other bacteria, mycobacteria are slowly growing, requiring at least 5 days of

incubation, with many requiring 1 or more weeks for visible growth; of note, some do not grow at all on solid media (1).

The *Mycobacterium* genus includes strict pathogens, potential or opportunistic pathogens, and nonpathogenic, saprophytic species. Gene sequence similarities within the genus (>94.3% for the 16S rRNA gene) and robust phylogenetic reconstructions using concatenated sequences of housekeeping genes have confirmed the natural division between slowly and rapidly growing mycobacteria and have also demonstrated that all slowly growing mycobacteria belong to a single evolutionary branch that emerged from the rapidly growing mycobacteria. This feature is intrinsically linked to their pathogenic ability to infect humans, and therefore, all strict pathogens and most opportunistic pathogens belong to the evolutionary branch of slowly growing mycobacteria (2–4). From 41 valid species in 1980, the genus *Mycobacterium* currently encompasses almost 200 recognized species and subspecies (2–6), many of which either can cause clinical disease, have been associated with disease, or have been isolated from clinical specimens without any known clinical correlation.

***Mycobacterium tuberculosis* Complex**

Tuberculosis (TB) is caused by a group of closely related, slowly growing mycobacteria collectively named the *Mycobacterium tuberculosis* complex, which infect a large spectrum of mammals, including humans. Infection occurs when a person (or animal) inhales 1- to 5-μm droplet nuclei containing tubercle bacilli that reach the alveoli of the lungs. Following exposure, either the *M. tuberculosis* complex can be killed by the host's immune system; active TB disease can occur in different areas of the body such as regional lymph nodes, lung, kidneys, brain, larynx, and bone; or latent TB infection (LTBI) may be established. Latency refers to the condition of chronic infection without clinical signs or symptoms of pulmonary TB (7). LTBI is significant because these patients serve as a major reservoir of the *M. tuberculosis* complex in the population and because these patients can develop active pulmonary TB if they become immunosuppressed.

The *M. tuberculosis* complex is comprised of *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), *Mycobacterium caprae*, *Mycobacterium africanum*, *Mycobacterium pinnipedii*, *Mycobacterium microti*, *Mycobacterium orygis*, *Mycobacterium mungi* (8), dassie bacillus (9), chimpanzee bacillus (10), and the rare, smooth-colony-morphology tubercle bacillus named *Mycobacterium canettii* (11). The *M. tuberculosis* complex shares identical matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) profiles, high-performance liquid chromatography (HPLC) patterns, and 16S rRNA gene sequences, with >99% identity at the nucleotide level for some species. However, despite these similarities, complex members differ significantly in morphology, biochemistry, host spectra, disease patterns in animals, antimicrobial susceptibility testing (AST) data, geographic ranges, and epidemiological patterns. The use of nucleic acid amplification tests (NAATs), including Amplified MTD (MTD; Hologic Inc., San Diego, CA) and Xpert MTB/RIF (Xpert; Cepheid, Sunnyvale, CA), cannot usually differentiate among members of this complex, although specific primers can be used to differentiate complex members with some sequencing methods. At present, there is only one commercial test, the Genotype MTBC line probe assay (Hain Lifescience, Nehren, Germany), that has the ability to differentiate among some of the members of the *M. tuberculosis* complex, such as *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, and *M. caprae*, from growth-positive cultures (broth or solid medium) or directly from AFB smear-positive, processed specimens (12).

***Mycobacterium tuberculosis*.** *M. tuberculosis* has killed more than 100 million people over the last 100 years (see references 13 and 14 for more detailed information regarding the pathophysiology, clinical manifestations, diagnosis, and treatment of TB). The global burden of TB is enormous. In 2015, an estimated 10.4 million people developed TB, and 1.8 million died, including HIV-infected individuals (15). In 2015, there were an estimated 1.2 million new TB cases in HIV-positive individuals (11% of all new cases of TB), 60% of whom were living in India, Indonesia, China, Nigeria, Pakistan,

and South Africa. Although the mortality rate from TB has fallen 22% between 2000 and 2015, TB is one of the top 10 causes of death worldwide.

In 2016, a total of 9,287 new TB cases were reported in the United States. This represents the lowest number of TB cases on record and a 2.7% decrease from the number in 2015. The 2016 TB incidence of 2.9 cases per 100,000 persons represents a slight decrease compared with the incidence in 2015. However, epidemiological modeling demonstrates that if similar, low rates of decline continue, the goal of U.S. TB elimination (<1 case/million population) will not be reached during this century (16).

Drug resistance among *M. tuberculosis* isolates can develop in two ways, with primary resistance occurring in persons who are initially exposed to and infected with resistant organisms. In contrast, secondary resistance, or acquired resistance, develops during TB therapy, either because the patient was treated with an inadequate regimen, because the patient did not take the prescribed regimen appropriately, or because of other conditions such as drug malabsorption, an inadequate potency of the compound, or drug-drug interactions that led to low serum levels. By the end of 2015, data on TB drug resistance were available for 155 countries, accounting for more than 95% of the world's population and estimated TB cases. Globally, an estimated 3.9% of new cases and 21% of previously treated cases have multidrug-resistant TB (MDR-TB), i.e., resistance to at least rifampin (RIF) and isoniazid (INH), and RIF-resistant TB; these levels have remained virtually unchanged in recent years. In 2015, there were an estimated 480,000 new cases of MDR-TB worldwide, with 100,000 RIF-resistant TB cases. India, China, and the Russian Federation accounted for 45% of the 580,000 combined cases. Extensively drug-resistant TB (XDR-TB) was reported by 117 countries by 2015. An estimated 9.5% of people with MDR-TB have XDR-TB. XDR-TB is defined as resistance to INH and RIF plus resistance to any fluoroquinolone (FQ) and at least one of three injectable second-line anti-TB drugs (i.e., kanamycin, capreomycin, or amikacin) (15).

***Mycobacterium bovis*.** *M. bovis* causes TB in warm-blooded animals, including cattle, deer, and elk, and can be transmitted to primates and humans. Up to 30% of TB cases in Europe were caused by *M. bovis* in the 1900s and were transmitted to humans largely via the inhalation of infectious droplets from infected cattle and the consumption of contaminated, unpasteurized dairy products (17). The introduction of milk pasteurization and cattle control programs in 1917 all but eradicated *M. bovis* from cattle and humans in most of the United States and other resource-rich nations. Fewer than 0.02% of U.S. cattle tested positive by a tuberculin skin test (TST) in 2002, and <0.002% of 377,000 cattle tested in a 2008 California *M. bovis* investigation were positive (18). A study of human TB cases in the United States from 1995 through 2005 estimated that only 1.4% of cases were still caused by *M. bovis*, most of which were among individuals born outside the United States, resulting in about 230 cases per year (19). However, more recently, in certain regions of the United States, such as along the border of Mexico, and in Mexican-born individuals in New York City (17), the prevalence *M. bovis* has been shown to be significantly higher than the national prevalence. In San Diego, CA, over 45% of all culture-confirmed TB cases in children and 8% of all TB cases in 2004 to 2007 were due to *M. bovis*, primarily due to the consumption of unpasteurized cheese products (20).

Isolates of *M. bovis* are intrinsically resistant to pyrazinamide (PZA) but may be susceptible to other drugs used for the treatment of TB. This is one reason why it is important to identify *M. tuberculosis* complex isolates to the species level. People at risk for acquiring *M. bovis* include individuals who work with cattle, bison, deer, or elk or products from these animals, such as hides, milk, or meat. People who drink raw (unpasteurized) milk or consume dairy products made from raw milk are also at a higher risk. People who might be at a higher risk for *M. bovis* infection should talk to their health care providers about whether they should be regularly screened for TB infection using either the TST or an interferon gamma (IFN-γ) release assay (IGRA) (21).

***Mycobacterium bovis* BCG.** The live, attenuated BCG strain of *M. bovis* is used for TB vaccination; of note, this strain is not pathogenic in most hosts. Vaccination is used in high-prevalence areas primarily to prevent disseminated disease in young children;

vaccination protocols for these young children result in a 60 to 80% decrease in the incidence of TB (22). BCG vaccination is not used in low-prevalence countries such as the United States and other industrialized countries. Instillation of BCG into the bladder can also be used to treat bladder cancer given its ability to act as a strong tumor-immunomodulatory therapy. However, some individuals treated for bladder cancer can develop infections with *M. bovis* BCG in different organs; of importance is the differentiation of *M. bovis* from *M. bovis* BCG (23). Disseminated BCG infection as a result of TB vaccination is a rare complication, with an incidence of 0.06 to 1.56 cases per million vaccinations, occurring exclusively in patients with immune deficits. However, in these cases, the prognosis is unfavorable; up to 70% of patients die, despite intensive antituberculosis treatment (24). The rapid differentiation of *M. bovis* and *M. bovis* BCG diseases from *M. tuberculosis* disease is crucial for optimizing treatment, contact investigation, and implementing additional control measures at various levels of the health care system.

Latent tuberculosis infection. About one-third of the world's population has LTBI. In the United States, the National Health and Nutrition Evaluation Survey (1999 to 2000) estimated the prevalence of LTBI among civilian, noninstitutionalized, and nonhomeless populations to be about 11.2 million (4.2%) individuals (25). In a more recent survey (2011 to 2012), the prevalence of LTBI was estimated to be 12.4 million (4.4%) individuals (26). People infected with *M. tuberculosis* have a lifetime risk of developing active TB of 10% (27). However, persons with compromised immune systems, such as people living with HIV, malnutrition, or diabetes, have a much higher risk of progression from LTBI to active disease. For example, individuals living with HIV and infected with *M. tuberculosis* have a 7 to 10% annual risk of developing active TB disease (28).

Mycobacterium leprae and *Mycobacterium ulcerans*

M. leprae and *M. ulcerans* are also notable mycobacterial pathogens. Since leprosy and Buruli ulcer are very distinct diseases, they are not included as NTM.

***Mycobacterium leprae*.** *M. leprae*, a very slowly growing *Mycobacterium* species, is the cause of leprosy, a chronic granulomatous disease that affects the skin, peripheral nervous system, and mucous membranes. Patients with leprosy are classified as having paucibacillary or multibacillary disease. Paucibacillary (low numbers of organisms) disease is the milder form and is characterized by one or more skin macules. Multibacillary (large numbers of organisms) leprosy is associated with symmetric skin lesions, nodules, plaques, thickened dermis, and frequent involvement of the nasal mucosa. Lesions may be AFB smear microscopy positive.

M. leprae cannot be grown by using routine bacteriologic media, commercial automated detection systems, or cell culture. However, the bacteria can be grown for research purposes in mouse footpads and, more recently, in nine-banded armadillos, which are also susceptible to leprosy. For the most part, leprosy is diagnosed based on clinical signs and symptoms (29), with most diagnoses being made clinically in combination with suggestive pathological findings of skin lesions, although molecular techniques may also play a diagnostic role (30, 31).

In 2015, the number of new cases worldwide was 174,608 (corresponding to a prevalence rate of 0.29 cases per 10,000 population, a decrease in the rate of detection of new cases from 0.32 per 10,000 population in 2014) (29). India reported the highest number of new cases (127,326, or 60% of new cases), followed by Brazil (26,395) and Indonesia (17,202). In 2015, 178 new cases occurred in the United States; 72% of these new cases were reported by California, Florida, Hawaii, Louisiana, New York, and Texas (30). Worldwide, 1 million to 2 million individuals are disabled because of leprosy, but with appropriate treatment, these individuals are considered free of active infection.

Person-to-person spread via inhalation of infectious droplets is the usual mode of transmission of *M. leprae*. In the past, patients in the United States with leprosy were taken care of in sanatoria; the last sanatorium for the management of leprosy closed in 1999, with federally supported outpatient clinics being available throughout the United States and Puerto Rico for treatment. Diagnostic assistance is available from the National

Hansen's Disease Programs in Baton Rouge, LA (30, 32). Multidrug therapy must be used as therapy for leprosy to prevent or slow the development of resistance. For example, RIF is combined with dapsone to treat paucibacillary leprosy, while RIF and clofazimine are now combined with dapsone to treat multibacillary leprosy (33).

***Mycobacterium ulcerans*.** *M. ulcerans* is a strict pathogen of humans. Disease ranges from a localized nodule or ulcer to widespread ulcerative or nonulcerative disease, including osteomyelitis. If untreated, severe limb deformities with contractures and scarring are common (1). In Africa, the disease is referred to as Buruli ulcer, while in Australia, it is called Bairnsdale ulcer. Nevertheless, disease mediated by *M. ulcerans* is the third most common mycobacterial disease in the world (34).

Evidence suggests that *M. ulcerans* may be transmitted through mild injury to the skin after exposure to contaminated environmental sources such as water or soil (35).

Although *M. ulcerans* has never been cultured directly from environmental waters that are associated with human infections and the mode of transmission is unknown (36), aquatic insects isolated from the wild in areas where Buruli ulcer is endemic can be naturally colonized with *M. ulcerans*, and aquatic insects infected in the laboratory are able to infect mice by biting (37). In humans, the unique pathogenic capacity of *M. ulcerans* is due to the secretion of a potent macrolide toxin, mycolactone, encoded by a plasmid that causes tissue damage and inhibits the immune response (38).

M. ulcerans needs a temperature of between 29°C and 33°C and a low (2.5%) oxygen concentration to grow. Since growth detection is difficult and has a low yield, molecular techniques are preferable for diagnosis (39–41). During a World Health Organization (WHO) meeting on Buruli ulcer in 2013 (42), a new recommendation for laboratory diagnosis was proposed: national control programs should strengthen laboratory confirmation of cases to ensure that at least 70% of all reported cases are laboratory confirmed by positive PCR.

It is difficult to determine the true incidence of disease caused by *M. ulcerans* since it is not easily isolated in laboratories. In 2015, according to the WHO, 2,037 new cases of *M. ulcerans* infection were reported globally in 13 countries, with the majority of cases being reported from West and Central Africa (34). Because *M. ulcerans* is most often associated with tropical wetlands, it is thought to proliferate in mud beneath stagnant waters (43). Individuals of all ages and both sexes may be infected; many are children under 15 years of age. Finally, overwhelming evidence indicates that 8 weeks of RIF-streptomycin or 4 weeks of RIF-streptomycin followed by 4 weeks of RIF-clarithromycin or 8 weeks of other oral regimens achieve recurrence-free healing of Buruli ulcer (44).

Nontuberculous Mycobacteria

Nontuberculous *Mycobacterium* spp. have been called a variety of names, from atypical mycobacteria to MOTT (mycobacteria other than TB), and today are most frequently called nontuberculous mycobacteria or NTM. For many years, the Runyon classification (45) of *Mycobacterium* was utilized in clinical laboratories to provide a convenient way to differentiate among NTM based on the rate of growth of the organism from subculture and pigment production. With the increasing use of molecular and other nontraditional biochemical means of identification, this classification system is no longer universally used and has limitations; however, most mycobacteriologists would agree that the differentiation of the NTM into those that are slowly growing NTM (requiring $>$7 days to produce mature colonies on solid media from a dilute inoculum under ideal conditions) and those that are rapidly growing NTM (producing mature colonies on solid media in $\leq$7 days when subcultured on appropriate media) remains useful. Table 1 lists more recently identified novel species of NTM. Many of the listed species have been isolated from rare cases of human disease, but with newer molecular methods, more of these mycobacteria may be recognized in clinical specimens and correlated with disease. Table 1 also provides information about growth rates (rapid or slow growth). Furthermore, species that are closely related to the

TABLE 1 List of novel NTM species described since 2011[a]

Novel species or subspecies (reference)[c]	Yr of description	Type of growth	Close relative(s)
M. abscessus subsp. *abscessus* (78)	2011	Rapid	*M. abscessus*
M. abscessus subsp. *bolletii* (78)	2016	Rapid	*M. abscessus*
M. abscessus subsp. *massiliense* (78)	2016	Rapid	*M. abscessus*
M. algericum (305)	2011	Rapid	*M. terrae* complex
M. alsense (306)	2016	Slow	*M. asiaticum*
M. anyangense (307)	2015	Rapid	*M. smegmatis-M. fortuitum*
M. arabiense (308)	2013	Rapid	*M. neoaurum-M. hodleri*
M. arcueilense (309)	2016	Rapid	*M. fortuitum* group, *M. septicum*, *M. farcinogenes*[b]
M. bourgelatii (310)	2013	Rapid	*M. intermedium*
M. celeriflavum (311)	2015	Rapid	*M. moriokaense*
M. engbaekii (312)	2013	Rapid	*M. hiberniae*
M. europaeum (313)	2011	Slow	*M. simiae*
M. fragae (314)	2013	Slow	*M. celatum*
M. franklinii (315)	2015	Rapid	*M. chelonae-M. abscessus*
M. helvum (316)	2016	Rapid	No relation to any characterized type strains of mycobacteria
M. heraklionense (312)	2013	Slow	*M. arupense*
M. hippocampi (317)	2014	Rapid	*M. flavescens-M. goodii*
"*M. icosiumassiliensis*" (318)	2016	Slow	*M. terrae* complex
M. iranicum (319)	2013	Rapid	*M. gilvum*
M. koreense (320)	2012	Slow	*M. triviale*
M. litorale (321)	2012	Rapid	*M. monacense*
M. longobardum (312)	2013	Slow	*M. terrae*
M. lutetiense (309)	2016	Rapid	*M. fortuitum* group, *M. septicum*, *M. farcinogenes*[b]
"*M. malmesburyense*" (322)	2016	Rapid	*M. moriokaense*
"*M. massilipolynesiensis*" (323)	2017	Rapid	*M. phlei*
M. minnesotense (324)	2013	Slow	*M. arupense*
M. montmartrense (309)	2016	Rapid	*M. fortuitum* group, *M. septicum*, *M. farcinogenes*[b]
M. oryzae (325)	2016	Rapid	*M. tokaiense*, *M. murale*
M. paraense (326)	2015	Slow	*M. interjectum*
M. paragordonae (327)	2014	Slow	*M. gordonae*
M. paraintracellulare (70)	2016	Slow	*M. intracellulare*
M. parakoreense (328)	2013	Slow	*M. koreense*
M. saopaulense (329)	2015	Rapid	*M. chelonae*, *M. abscessus*
M. sarraceniae (316)	2016	Rapid	No obvious relation to any characterized type strains of mycobacteria
M. sediminis (308)	2013	Rapid	*M. neoaurum*, *M. hodleri*
M. sherrisii (330)	2011	Slow	*M. simiae*
M. shinjukuense (331)	2011	Slow	*M. tuberculosis* complex, *M. marinum*, *M. ulcerans*
"*M. virginiense*" (332)	2016	Slow	*M. terrae* complex
M. yongonense (71)	2013	Slow	*M. intracellulare*

[a]See reference 6.
[b]*M. farcinogenes* is also a slowly growing mycobacterium, although some rapidly growing mycobacteria are genetically related to this species.
[c]Species in quotation marks are proposed names and do not yet have a standing in nomenclature.

novel species are also listed, which may be helpful for the identification of an isolate under study.

The plethora of newly described species seen in the last few decades is in part the consequence of the availability and increased reliability of new DNA sequencing methods that can differentiate even closely related species and in part the consequence of an increased frequency of isolation of mycobacteria (46–50). The latter may be the result of newly emerging, human-made reservoirs (e.g., treated urban water and sewage systems, swimming pools, hot tubs, pedicure footbaths, showers, and medical devices) for certain species. Other species, like *Mycobacteria gordonae*, are common in both natural and artificial sources. NTM can form biofilms on a wide range of organic (plastic, silicone, rubber, and PVC) and inorganic (glass, metals, and metallic fluids of machines) materials due to their hydrophobic cell wall and their resistance to disinfectants, antibiotics, or heavy metals. In both natural and human-made environments, biofilms may have an important role in protecting NTM against aggressive external factors and promoting their colonization. This colonization by NTM in biofilms may lead to contamination that can be a source of either pseudoinfections or true NTM diseases. Pseudoinfections may be the result of contamination during the collection of speci-

TABLE 2 Clinical and microbiological criteria for diagnosis of nontuberculous mycobacterial lung disease[a]

Criterion type	Description
Clinical	(i) Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or a high-resolution computed tomography scan that shows multifocal bronchiectasis with multiple small nodules and (ii) appropriate exclusion of other diagnoses
Microbiological	(i) Positive culture results from at least 2 separate expectorated sputum samples, and if the results from 2 sputum samples are nondiagnostic (i.e., culture negative), consider repeat sputum AFB smears and cultures; (ii) positive culture result from at least 1 bronchial wash or lavage specimen; or (iii) transbronchial or other lung biopsy specimen with mycobacterial histopathological features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy specimen showing mycobacterial histopathological features (granulomatous inflammation or AFB) and 1 or more sputum or bronchial wash specimens that are culture positive for NTM
Patient management	(i) Expert consultation should be obtained when NTM that either are infrequently encountered or usually represent environmental contamination are recovered; (ii) patients who are suspected of having NTM lung disease but do not meet the diagnostic criteria should be monitored until the diagnosis is firmly established or excluded; and (iii) making the diagnosis of NTM lung disease does not, *per se*, necessitate the institution of therapy, which is a decision based on potential risks and benefits of therapy for individual patients

[a]Adapted from reference 52 with permission of the American Thoracic Society (copyright © 2007 American Thoracic Society).

mens (e.g., biofilms in improperly cleaned endoscopes) (51) or contamination during laboratory testing (e.g., a contaminated water source for reagent preparation). Pseudoinfections may be recognized by an increase in the number of AFB smear-positive specimens, an increase in the frequency of detection of a particular NTM species, or an increase in the prevalence of a peculiar AST pattern. While pseudoinfections do not necessarily cause disease, they can create difficult diagnostic dilemmas (52, 53).

According to the American Thoracic Society (ATS)/Infectious Diseases Society of America (IDSA) 2007 statement (52), when NTM are suspected as the etiology of disease, definitive diagnosis should always be supported by repeated isolation of NTM from several specimens of the patient or a single specimen if it is collected aseptically from a sterile body site. However, laboratory identification of potentially pathogenic or saprophytic NTM alone is not enough to direct patient care. Laboratory results should always be correlated with the individual's clinical presentation and radiological and histological findings (Table 2) to determine the clinical significance, if any.

For the purposes of this article, this group of organisms is divided into two major groups, i.e., slowly growing NTM and rapidly growing NTM.

Slowly growing nontuberculous mycobacteria frequently involved in human disease. NTM may result in colonization, infection, and/or disease (52, 54). Colonization and infection can be transient, intermittent, or prolonged. Since humans are in regular contact with NTM in the environment, NTM can be detected in the respiratory and gastrointestinal tracts or on the skin of healthy individuals.

NTM may grow in natural and human-made environments, such as treated urban water and sewage systems; swimming pools; hot tubs; pedicure footbaths; showers; tattoo inks; fish tanks; or medical devices such as endoscopes and their washing machines and heater-cooler devices, ice machines used to refrigerate surgical solutions, and inadequately sterilized surgical equipment or solutions (52, 54). Certain NTM such as *Mycobacterium avium* or *Mycobacterium marinum* are more commonly recoverable from artificial sources, while the natural reservoir of *Mycobacterium kansasii* and *Mycobacterium xenopi* is currently unknown.

Most NTM are ubiquitous environmental microorganisms that can be recovered from soil and both treated and untreated freshwater and seawater (2, 3, 5, 52, 55). Since NTM may be found in both natural and human-made reservoirs, human infections are suspected to be acquired from these environmental sources. However, the identification of the specific source of infection is usually not possible. NTM diseases are not reportable diseases in many public health jurisdictions since they are not considered communicable; therefore, surveillance data are limited and unreliable (52, 54).

Marras et al. (47) reported an increase in the rate of detection of pulmonary NTM isolates in Ontario, Canada, from 9.1 cases per 100,000 persons in 1997 to 14.1 cases per 100,000 persons in 2003. In a follow-up study (48), that same research group measured

the prevalence and temporal trends of pulmonary NTM disease among residents of Ontario, Canada, from 1998 to 2010. The 5-year prevalence increased from 29.3 cases per 100,000 persons from 1998 to 2002 to 41.3 cases per 100,000 persons in 2006 to 2010.

Adjemian et al. (50) described the prevalence and trends of pulmonary NTM disease among adults aged 65 years or older throughout the United States. From 1997 to 2007, the annual incidence significantly increased from 20 to 47 cases/100,000 persons, or 8.2% per year. Women were 1.4 times more likely to be diagnosed with pulmonary NTM than men. Relative to whites, Asians/Pacific Islanders were twice as likely to be infected, whereas blacks were half as likely.

(i) *Mycobacterium avium* complex. Pulmonary infection caused by the *Mycobacterium avium* complex (MAC) can occur in immunocompetent hosts; disseminated infections usually occur in people living with HIV. The most common presentations of MAC lung infections in immunocompetent hosts are TB-like apical fibrocavitary disease or interstitial nodular infiltrates and bronchiectasis. MAC can also be associated with hypersensitivity pneumonitis, which is also known as "hot tub lung." This syndrome is a combination of inflammatory reactions and infectious mechanisms and is the result of exposure to contaminated indoor spas or showers. In children, cervical lymphadenitis is the most common presentation (56).

The MAC is frequently isolated in laboratories in the United States. Diseases caused by the MAC are not consistently reportable throughout the United States (57). Disseminated MAC infection has been associated with people living with HIV with low CD4 counts, but since the introduction of highly active antiretroviral therapy, the incidence in this population is declining. The MAC is acquired from the environment and is not thought to be spread from person to person. Recently, *Mycobacterium chimaera*, a species within the MAC, was implicated in causing disease from the use of contaminated heater-cooler devices used in open heart surgery (58–60).

Strollo et al. (61) reported that state-specific numbers of cases and costs are critical for quantifying the burden of pulmonary NTM disease in the United States. Available direct-cost estimates of NTM disease-related medical encounters were applied to NTM disease prevalence estimates derived from Medicare beneficiary data (2003 to 2007), and persons younger than 65 years of age were included, with case estimates based on the age distribution of cases from a reported survey of NTM treatment (62). In 2010, those authors estimated that there were 86,244 national cases, totaling to $815 million in costs for disease management. Costs of medical encounters among individuals aged 65 years and older ($562 million) were 2-fold higher than those for individuals younger than 65 years of age ($253 million). Projected 2014 estimates resulted in 181,037 national annual cases ($1.7 billion in costs).

The members of the MAC are slowly growing mycobacteria with smooth, flat, and transparent colonies. The MAC is one of the NTM that is most commonly identified as a pathogen in respiratory specimens. Historically, the MAC included two species, *M. avium* and *Mycobacterium intracellulare*. For the *M. avium* species, four subspecies have been assigned, *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum* (63).

M. avium subsp. *avium* is associated with avian TB, while *M. avium* subsp. *hominissuis* is the etiological agent of disseminated disease in humans (especially in AIDS patients with low CD4 counts), chronic lung diseases in patients with cystic fibrosis (CF) or bronchiectasis, or cervical lymphadenitis in children (63–66). Infections with *M. avium* subsp. *paratuberculosis* result in a chronic granulomatous enteric infection in cattle (Johne's disease) and may have a similar role in Crohn's diseases in humans (67, 68). *M. avium* subsp. *silvaticum* has been isolated from wood pigeons, a crane, a penguin, roe deer, and a hazel hen; however, due to the lack of a reliable method to identify this organism until recently, there has been a lack of data regarding its pathogenicity and zoonotic potential (69). *M. intracellulare* is primarily a respiratory pathogen in humans, and an association with disseminated diseases is less common (63).

Molecular identification studies have revealed numerous additional sequence

variants within the MAC, of which *Mycobacterium colombiense*, *M. chimaera*, *Mycobacterium vulneris*, *Mycobacterium marseillense*, *Mycobacterium timonense*, *Mycobacterium arosiense*, *Mycobacterium yongonense*, *Mycobacterium paraintracellulare*, and *Mycobacterium bouchedurhonense* were recently elevated to the species level (52, 54, 70–72). The standard treatment of MAC disease includes macrolides (clarithromycin or azithromycin), ethambutol (EMB), and a rifamycin (RIF or rifabutin). If a more aggressive therapy is required, an injectable aminoglycoside (streptomycin or amikacin) may be added to this combination (52, 54).

(ii) *Mycobacterium kansasii*. *M. kansasii* is a slowly growing photochromogenic mycobacterium. *M. kansasii* is the second most common cause of NTM disease in some regions of the United States, England, Wales, and France (64). The isolation of the bacteria in human specimens is almost always associated with disease. The major reservoir of the bacteria is likely to be tap water. The primary manifestation of *M. kansasii* infection resembles that of pulmonary TB, with cavitary infiltrates in the upper lobes. However, noncavitary and nodular-bronchiectatic lung disease has also been observed. Risk factors include pneumoconiosis (especially in miners), chronic obstructive lung disease, malignancy, and alcoholism. Disseminated infections can be observed in patients with impaired cellular immunity. The recommended therapy for *M. kansasii* infections is INH, RIF, and EMB. *M. kansasii* also shows low MIC values for clarithromycin, amikacin, and FQ (52, 54).

(iii) *Mycobacterium xenopi*. Clinical and radiological disease manifestations of *M. xenopi* infection vary according to the patient's immunological status and can be classified into three groups: a cavitary form in patients with preexisting pulmonary disease, a solitary nodular form in immunocompetent patients, and an acute infiltrate form in immunosuppressed patients (73). *M. xenopi* lung disease usually develops in individuals with chronic obstructive pulmonary disease or bronchiectasis with an apical cavitary radiological appearance. Extrapulmonary cases with joint and soft tissue infections have also been observed.

M. xenopi, a slow-growing thermophilic mycobacterium with an optimal growth temperature of 42°C to 45°C, was first isolated from skin lesions of *Xenopus laevis*, an African toad. It can frequently be recovered from hot water systems, especially in hospitals, which may also lead to nosocomial (hospital) infections or pseudoinfections (contamination of clinical specimens or bronchoscopes during collection). *M. xenopi* is second to the MAC as a cause of NTM lung disease in Canada, the United Kingdom, and certain parts of Europe. The optimal treatment regimen for *M. xenopi* includes clarithromycin or azithromycin, EMB, and RIF (52, 54).

(iv) *Mycobacterium malmoense*. *M. malmoense* was first isolated from a patient in Malmö, Sweden. *M. malmoense* is usually recovered from sputum or cervical lymph nodes of children or adults with underlying chronic pulmonary disease, often with cavitary involvement; to date, most of the isolates have been reported from northern Europe, Zaire (Democratic Republic of Congo), and Japan. The numbers of infections with *M. malmoense*, however, may be underestimated since growth detection is long, requiring up to 8 to 12 weeks of incubation. Of significance, treatment of pulmonary *M. malmoense* infection may be complicated. The recommended therapy includes INH, RIF, and EMB with or without macrolides and FQ (52, 54).

(v) *Mycobacterium haemophilum*. *M. haemophilum* is a fastidious mycobacterium with an optimal growth temperature of 28°C to 30°C that grows better on solid medium and requires hemin or hemoglobin as an iron source. Due to these special growth conditions, this organism is often underrecognized. The temperature preference is in line with the fact that *M. haemophilum* usually infects cooler body sites such as the extremities. Most infections are reported in AIDS patients. Additional cases are associated with other immunosuppressed patients, especially those with solid-organ transplants, bone marrow recipients, or long-term steroid users. The classical clinical presentation is multiple skin lesions or ulcerations appearing on the extremities that are occasionally associated with abscesses, fistulas, or osteomyelitis. Infections were also identified in immunocompetent children with cervical lymphadenitis and in a patient

with a pulmonary nodule. The optimal therapy for *M. haemophilum* is unknown. Regimens may include clarithromycin, rifamycins (RIF and rifabutin), and FQ (52, 54).

(vi) *Mycobacterium genavense*. *M. genavense* is a fastidious, slowly growing mycobacterium that shows better recovery in liquid medium supplemented with mycobactin J, especially at an acidic pH (pH 5.5). It was first isolated from the blood of a patient with AIDS in Geneva, Switzerland. Clinical isolation of *M. genavense* is usually associated with HIV-related or other causes of immunosuppression. This mycobacterium was recovered from AIDS patients with disseminated diseases; individuals with lymphadenitis; bone marrow, liver, spleen, stool, and blood samples; and individuals with genital and soft tissue infections. *M. genavense* is one of the most common mycobacterioses in pet birds (74). Susceptibility testing of *M. genavense* is complicated by the fact that the bacterium is difficult to grow and shows preferable growth at an acidic pH. An optimal therapy has not been determined, but therapy may include clarithromycin, rifamycins, FQ, and aminoglycosides (amikacin) (52, 54).

(vii) *Mycobacterium marinum*. *M. marinum* is a photochromogenic, slowly growing (8 to 12 days) organism that optimally grows at temperatures of between 30°C and 33°C. *M. marinum* is the causative agent of "swimming pool" or "fish tank" granuloma, which is the result of finger, hand, arm, elbow, knee, or toe soft tissue injury in freshwater or saltwater due to punctures from fins of fish or shrimp or from cleaning of fish tanks or swimming pools. However, proper chlorination significantly decreased its colonization in swimming pools. The typical presentation is a single papule on an extremity that may progress to a shallow ulcer, usually 2 to 3 weeks after inoculation. Severe complications may also include osteomyelitis of the bones and tenosynovitis and arthritis of the joints. Treatment may include surgical debridement and treatment with clarithromycin and EMB or EMB and RIF. In cases of deep-structure involvement, a combination of clarithromycin, EMB, and RIF can be considered (52, 54).

(viii) *Mycobacterium szulgai*. *M. szulgai* is a slowly growing mycobacterium that is scotochromogenic at 37°C and photochromogenic at 25°C (75). Recovery of *M. szulgai* from the environment is rare and very unusual. Therefore, similar to *M. kansasii*, isolation of the bacteria in clinical laboratories almost always has clinical significance. The clinical presentation of pulmonary infections with *M. szulgai* is indistinguishable from that of TB and usually develops in middle-aged men with alcohol abuse, smoking, and chronic obstructive pulmonary disease. Other extrapulmonary clinical manifestations may include cervical lymphadenitis, cutaneous infections, osteomyelitis, tenosynovitis, bursitis, or disseminated infections in both AIDS patients and immunocompetent patients. *M. szulgai* shows low MIC values for most antituberculosis drugs; therefore, a three- to four-drug regimen may be recommended (52, 54).

(ix) *Mycobacterium scrofulaceum*. *M. scrofulaceum* is a slowly growing mycobacterium that is scotochromogenic at 25°C and 37°C. Its name is derived from the histological term "scrofula," used to describe infections of the cervical lymph nodes by mycobacteria. Wolinsky documented a change from *M. scrofulaceum* to MAC as the common cause of mycobacterial cervical lymphadenitis in children in the 1980s (76). AST data are not available, and standardized treatment regimens for *M. scrofulaceum* have not been determined. Confirmed disease-causing isolates of *M. scrofulaceum* should have AST performed on them (52).

Rapidly growing nontuberculous mycobacteria frequently involved in human disease. Many newly recognized species belonging to the genus *Mycobacterium* are rapidly growing mycobacteria such that these organisms constitute approximately half of the currently recognized/validated mycobacterial species. Rapidly growing mycobacteria not only represent many species but also are responsible for a range of disease manifestations in the human host, vary widely in their AST profiles, and reside in a vast number of environmental reservoirs.

Rapidly growing NTM are very commonly isolated in clinical laboratories and can be the cause of significant local and disseminated diseases. A review of all rapidly growing mycobacteria is beyond the scope of this document; only those most commonly implicated in human infection are briefly discussed here.

(i) *Mycobacterium abscessus* and its subspecies. *M. abscessus* and its subspecies are often isolated from clinical specimens from CF patients. These organisms are environmental, being found worldwide in water, soil, and dust, and are responsible for a wide variety of infections. These organisms often cause infections of skin and soft tissue, but they can cause more serious infections, including disseminated disease. Severe lung infections can occur in persons with underlying chronic lung disease, including patients with CF. Other individuals with underlying respiratory conditions or impaired immune systems can be at risk for lung infections. Skin infections are often caused by injections of substances contaminated with *M. abscessus*, via invasive medical procedures when contaminated equipment is utilized, or by pedicures (77).

Until recently, there was no evidence of human-to-human or animal-to-human transmission of NTM. However, two recent reports investigating outbreaks in CF patients using thorough conventional epidemiological and state-of-the-art molecular typing investigations, such as whole-genome sequencing, indicated the potential transmission of *M. abscessus* (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense* [78]) among these patients (79, 80). These results have challenged the dogma of the lack of person-to-person transmission of NTM.

Two studies from the United Kingdom (80, 81) utilized whole-genome sequencing and epidemiological analyses to assess the potential for person-to-person transmission of *M. abscessus* among CF patients. First, a retrospective study by Bryant et al. (80) examined the genetic relatedness of 168 *M. abscessus* isolates from 31 adult CF patients obtained during 2007 to 2011. Overall, those researchers concluded that person-to-person transmission of *M. abscessus* within the hospital was a plausible explanation for their phylogenomic and epidemiological observations. A second, more recent study by Harris et al. (81) employed an approach similar to that of Bryant et al. to test for potential cross-infection among a cohort of pediatric CF patients. Those researchers performed whole-genome sequencing on 27 *M. abscessus* isolates from 20 pediatric patients. Their epidemiological data, however, did not identify opportunities for transmission between patients, except for one sibling pair who lived in the same household. That study did not include environmental sampling within the hospital and, therefore, could not rule out a common source of infection on-site. Their conclusion was that the collective study data did not suggest cross-transmission of *M. abscessus* between pediatric CF patients.

Although the taxonomy of organisms belonging to the *Mycobacterium abscessus* complex has been somewhat controversial, whole-genome sequencing data strongly support the presence of three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense* (78). *M. abscessus*, a nonchromogenic and rapidly growing mycobacterium, can be recovered from environmental sources and is the most pathogenic and chemotherapy-resistant rapidly growing mycobacterium. Human diseases include pulmonary infections and skin, soft tissue, and bone infections that usually develop following trauma or surgery or postinjection. *M. abscessus* is a frequently isolated respiratory NTM, after the MAC, and accounts for more than 80% of all rapidly growing mycobacterial respiratory infections. Patients with lung disease due to *M. abscessus* are usually white, female nonsmokers older than 60 years of age without predisposing factors. Predisposing conditions that are commonly associated with *M. abscessus* pulmonary infections are bronchiectasis with reticulonodular lung infiltration, CF, and, rarely, lipoid pneumonia and gastrointestinal disorders with chronic vomiting. Patients with these predisposing factors are usually younger than 40 years of age. Approximately 80% of *M. abscessus* subsp. *abscessus* isolates are resistant to clarithromycin by an inducible *erm*(41) gene (82). Of note, this organism can also possess an additional *erm*-like gene, efflux pumps, an aminoglycoside 2′-*N*-acetyltransferase, and 12 other homologs of aminoglycoside phosphotransferases (83); therefore, treatment of diseases caused by this bacterium is often very challenging, and cure is rare unless the disease is limited or there is a focal disease presentation. Curative therapy may include surgical resection of focal (lung) involvements and/or combined multidrug therapy with macrolides and amikacin,

cefoxitin, or imipenem. *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* (a rare cause of human infection) contain an inducible erythromycin methylase *erm* gene that confers resistance to macrolides; therefore, these drugs should always be given in combination therapy (52, 54). The correct identification of the subspecies of *M. abscessus* is important, as *M. abscessus* subsp. *massiliense* is known to possess a nonfunctional *erm* gene, and as such, macrolide therapy may be effective (84, 85).

(ii) *Mycobacterium chelonae* complex. The *M. chelonae* complex consists of *M. chelonae* and three additional validated species (*Mycobacterium franklinii*, *Mycobacterium immunogenum*, and *Mycobacterium salmoniphilum*) (52). *M. chelonae*, a key human opportunistic pathogen, is a nonchromogenic, rapidly growing mycobacterium that is widely recoverable from human-made environments such as tap water or from freshwater and seawater. The most common clinical manifestations are skin, soft tissue, and bone infections, often related to infected piercing wounds, contaminated tattoo inks, plastic surgery, or liposuction. Disseminated diseases have been described in immunocompromised individuals, especially in those receiving high-dose steroids. Infections may also be associated with ophthalmic surgery or contact lens wear (keratitis). Pulmonary infections by *M. chelonae* are less common than are those by *M. abscessus* (86). Regimens for the treatment of *M. chelonae* infections may include tobramycin (for *M. chelonae*, tobramycin is more active than amikacin), clarithromycin, linezolid, imipenem, or clofazimine. To date, the Clinical and Laboratory Standards Institute (CLSI) has not addressed susceptibility testing for clofazimine, and as such, there are no standardized breakpoints or quality measures available. Usually, clarithromycin can be combined with a second agent to prevent the development of macrolide resistance. The removal of foreign bodies is also essential (52, 54).

(iii) *Mycobacterium fortuitum* complex. *M. fortuitum* is an environmental, nonchromogenic, and rapidly growing mycobacterium. The *M. fortuitum* complex includes the species *M. fortuitum*, *Mycobacterium peregrinum*, *Mycobacterium senegalense*, *Mycobacterium setense*, *Mycobacterium septicum*, *Mycobacterium porcinum*, *Mycobacterium houstonense*, *Mycobacterium boenickei*, *Mycobacterium brisbanense*, and *Mycobacterium neworleansense*. *M. fortuitum* is frequently associated with skin, soft tissue, and bone infections, while it rarely causes pulmonary disease, except in cases of lipoid pneumonia, gastroesophageal disorders, or disseminated diseases. Skin and soft tissue infections are common after mammoplasty and similar plastic surgery interventions or cardiac surgery (sternal wound infections). Footbaths in pedicure salons have also been identified as a source of *M. fortuitum*-associated furunculosis. *M. fortuitum* infections can usually be treated with a two-drug regimen based on *in vitro* susceptibility testing. This regimen may include FQ, doxycycline, amikacin, or sulfonamides. Although most *M. fortuitum* isolates are resistant to clarithromycin, certain members of the *M. fortuitum* complex, namely, *Mycobacterium peregrinum* and *Mycobacterium senegalense*, do not contain an inducible erythromycin methylase *erm* gene that confers resistance to macrolides (87). The removal of foreign bodies such as breast implants is also essential to clear infections (52, 54).

REGULATORY REQUIREMENTS AND GUIDELINES FOR TESTING MYCOBACTERIA

Introduction

The following section covers many of the regulatory aspects and best practices to address when testing for mycobacteria, especially for the *M. tuberculosis* complex, in the clinical laboratory.

Safety considerations are paramount when working with *M. tuberculosis* complex organisms. Prior to the passage of the Occupational Safety and Health Act (OSHA) in 1970, the employer had the sole responsibility of providing a safe workplace. Safety regulations and laws now come from federal, state, and local governments, and accrediting organizations and the Centers for Medicare and Medicaid Services (CMS) cover safety. Additional details on biosafety are covered in Biosafety, below.

TABLE 3 CLIA-approved proficiency testing programs[b]

Test	Proficiency testing performed by program[c]:						
	AAB	API	CAP	WSLH	MPEP (TB only)	Accutest	AAFP-PT
CLIA approved for 2017	Yes	Yes	Yes	Yes		Yes	Yes
Acid-fast stain	Yes	Yes	Yes	Yes		Yes	Yes
Mycobacteriology identification		Yes	Yes	Yes		Yes	
Mycobacteriology antimicrobial susceptibility testing		Yes	Yes		Yes[a]	Yes	
Molecular detection of *M. tuberculosis* complex and rifampin resistance			Yes[a]	Yes[a]	Yes[a]		

[a]Ungraded (educational) at this time.
[b]See reference 333.
[c]AAB, American Association of Bioanalysts; API, American Proficiency Institute; CAP, College of American Pathologists; WSLH, Wisconsin State Laboratory of Hygiene; MPEP, Model Performance Evaluation Program (Centers for Disease Control and Prevention); AAFP-PT, American Academy of Family Physicians proficiency testing program.

Clinical Laboratory Improvement Amendments

The regulation of U.S. clinical laboratories began after the Medicare and Medicaid laws went into effect in 1966. The Clinical Laboratory Improvement Amendments (CLIA) of 1967 established minimum standards for Medicare-participating laboratories engaged in interstate commerce (i.e., only a relatively small portion of clinical laboratories). In the 1980s, intensive media coverage of poor cytology laboratory practices, especially in the reading of Papanicolaou smears, and charges of lax enforcement of federal regulations contributed to the passage of the 1988 CLIA and the regulations that now define standards of cytology laboratory practice in the United States (88). The 1988 CLIA require that all laboratories performing human clinical testing have a certificate issued by the CMS, bringing all laboratories performing this testing under federal regulations. The 1988 CLIA regulations (final publication in 1992) are based on test method complexity: waived complexity, moderate complexity, and high complexity. The requirements become more stringent as the testing becomes more complex. The CLIA specify quality standards for proficiency testing, patient test management, quality control, personnel qualifications, and quality assurance for laboratories performing moderate- and/or high-complexity tests. The CLIA cover approximately 251,000 laboratory entities (89).

Also, two U.S. states, New York (90) and Washington (91), have state licensure programs that have exemption from CLIA program requirements (92). These states have inspection programs that the CLIA deemed at least as rigorous as a CLIA inspection.

All laboratories must be certified to perform testing on human specimens under CLIA (89). If the laboratory provides services in the subspecialty of mycobacteriology, the laboratory must meet the CLIA requirements specified in Title 42 Code of Federal Regulations (CFR) 493.1230 through 493.1256, 493.1262, and 493.1281 through 493.1299. The 1988 CLIA differentiate five types of laboratories for proficiency testing purposes: (i) those that interpret acid-fast stains and refer specimens to another laboratory appropriately certified in the subspecialty of mycobacteriology; (ii) those that interpret acid-fast stains, perform primary inoculation, and refer cultures to another laboratory appropriately certified in the subspecialty of mycobacteriology for identification; (iii) those that interpret acid-fast stains and isolate and perform identification and/or AST of *M. tuberculosis* but refer other mycobacterial species to another laboratory appropriately certified in the subspecialty of mycobacteriology for identification and/or AST; (iv) those that interpret acid-fast stains and isolate and identify all mycobacteria to the extent required for correct clinical diagnosis but refer AST to another laboratory appropriately certified in the subspecialty of mycobacteriology; and (v) those that interpret acid-fast stains, isolate and identify all mycobacteria to the extent required for correct clinical diagnosis, and perform AST on the organisms isolated. For institutions in the United States that offer proficiency testing programs for mycobacteriology, see Table 3.

As of 1 January 2016, an "individualized quality control plan" (IQCP) is an alternate quality control option to Title 42 CFR 493.1250. Instead of the CLIA quality control regulatory requirements, an IQCP may be implemented to meet CLIA quality control compliance, permitting the laboratory to customize its quality control plan according to the test method and use, environment, and personnel competency while providing equivalent quality testing; however, the laboratory cannot do less quality control than the manufacturer requires (93).

College of American Pathologists Requirements

The College of American Pathologists (CAP) has requirements pertaining to laboratory testing for mycobacteria (94). These include collection, transport and handling, reporting of results, medium/stain/reagent quality control (QC), controls and standards, AFB smear staining, the use of rapid methods for identification, inoculation on appropriate media, and safety considerations. The following turnaround times for specific tests are recommended: within 24 h of receipt for AFB smears and within 28 days for conventional AST results for the *M. tuberculosis* complex.

International Organization for Standardization 15189 Standard

International standards for clinical laboratories include the International Organization for Standardization (ISO) 15189 standard (95), which specifies the quality management system requirements particular to medical laboratories. It was developed by the ISO and last updated in 2012. While the ISO 15189 standard is based on the ISO/International Electrotechnical Commission 17025 standard (96) and the ISO 9001 standard (97), it is a unique document that takes into consideration the specific requirements of the medical environment and the importance of the medical laboratory to patient care. In the United States, both the CAP and the American Association for Laboratory Accreditation accredit to this international standard.

Statements Related to Mycobacterial Diseases from Various Professional Societies and Agencies

Official American Thoracic Society/Infectious Diseases Society of America/Centers for Disease Control and Prevention Clinical Practice Guidelines: Diagnosis of Tuberculosis in Adults and Children. In the 2017 official statement of the ATS, the IDSA, and the Centers for Disease Control and Prevention (CDC), endorsed by the European Respiratory Society in 2016 (98), diagnostic testing for TB was described. There are 23 evidence-based recommendations about diagnostic testing for LTBI, pulmonary TB, and extrapulmonary TB.

An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases. *An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases* (52) (under revision) provides, among other topics, clinical and microbiological criteria for diagnosing pulmonary NTM (Table 2). Key laboratory features of NTM health care and hygiene-associated disease prevention, prophylaxis, and treatment of NTM disease are described.

US Cystic Fibrosis Foundation and European Cystic Fibrosis Society Consensus Recommendations for the Management of Non-Tuberculous Mycobacteria in Individuals with Cystic Fibrosis. The U.S. Cystic Fibrosis Foundation and the European Cystic Fibrosis Society convened a panel of 19 experts to develop consensus recommendations for the screening, investigation, diagnosis, and management of NTM pulmonary disease in individuals with CF. A series of pragmatic, evidence-based recommendations as an initial step in optimizing management for this challenging condition was generated (99).

World Health Organization. The WHO produces numerous guidelines and policies regarding TB, Buruli ulcer, and leprosy diagnosis, along with biosafety recommendations; these can be accessed at www.who.org.

Healthy People 2020. *Healthy People 2020*, published in 2010 by the U.S. Department of Health and Human Services (DHHS), provides science-based, 10-year national objectives for improving the health of all Americans. Achieving these objectives is dependent

TABLE 4 Tests and turnaround times to be tracked by the laboratory[a]

Result	Recommended turnaround time
AFB smear (negative and positive)	Report AFB smear results within 24 h of specimen receipt
Positive TB NAAT	Report within 48 h of specimen receipt
New *M. tuberculosis* complex-positive culture results from any specimen source	Report culture identification of *M. tuberculosis* complex within ≤21 days of specimen receipt
M. tuberculosis complex first-line AST results on isolates that demonstrate resistance to any of the first-line drugs	Report first-line AST result within ≤28 days of specimen collection

[a]See references 242 and 334.

in part on the ability of health agencies at all levels of the government and on nongovernmental organizations to assess objective progress. For 3 decades, Healthy People has established benchmarks and monitored progress over time to encourage collaborations across communities and sectors, empower individuals toward making informed health decisions, and measure the impact of prevention activities. Healthy People is the basis for state and territorial health planning in many jurisdictions.

There is only one objective pertaining to the TB laboratory in *Healthy People 2020* (100): to increase the proportion of culture-confirmed TB patients with a positive nucleic acid amplification test (NAAT) result reported within 2 days of specimen collection (Immunization and Infectious Diseases 32). In 2008, the baseline year, 32% of culture-confirmed TB patients with a positive NAAT result had their test results reported within 2 days of specimen collection; the new goal is to achieve 77% by the year 2020. In 2015, the percentage for this objective was 46% (101).

Centers for Disease Control and Prevention

Metrics. To reduce transmission and to prevent further acquired TB, the CDC recommends that laboratories monitor the turnaround time by tracking the date/time of collection of specimens to the date/time of receipt by the laboratory. In addition, the length of time for generating and reporting results concerning AFB smear, NAAT, growth detection, identification, and AST results should be monitored (see Table 4 for tests and turnaround times) (102, 103): within the health care system, rapid transportation of specimens or positive AFB cultures to the laboratory either via courier or overnight with next-day delivery is encouraged.

Import permit. The CDC's Import Permit Program regulates the importation of infectious biological agents, infectious substances, and vectors of human disease into the United States, including *M. tuberculosis*. Prior to the issue of an import permit, the CDC reviews all applications to ensure that entities have appropriate safety measures in place for working safely with these important materials. Upon review of the application for an import permit, the CDC will contact the facility if an on-site inspection is required to evaluate whether the importer's biosafety measures are commensurate with the hazard posed by the infectious biological agent, infectious substance, and/or vector and the level of risk given its intended use (104).

International Air Transport Association and U.S. Department of Transportation Packaging and Shipping Requirements

Federal and international regulations must be met when using the U.S. Postal Service (USPS) or a commercial courier service to send specimens containing etiological agents, including *M. tuberculosis*. Proper packaging reduces the number of broken specimens, contains and absorbs leaking specimens, and helps ensure the safety of personnel handling them (103). Additional information about shipping of specimens/isolates of mycobacteria include Title 49 CFR Transportation Parts 100 and 185, available electronically and from the U.S. Government Printing Office (http://www.access.gpo.gov/); International Air Transport Association (IATA) dangerous-goods regulations (http://www.iata.org/); USPS regulations on hazardous materials, Domestic Mail Manual C023 (http://pe.usps.gov/); and information from the U.S. Department of Transportation (DOT) (http://www.dot.gov/), the Transportation Safety Institute (http://www.tsi.dot.gov/), and the International Civil Aviation Organization (http://www.icao.int/).

State Requirements

California and New York, among other states, have additional specific requirements for AFB testing and reporting of mycobacteriology results. The following states have their own licenses/permits for the performance of medical laboratory testing for specimens originating from a health care facility from that state: California, Florida, Maryland, New York, Pennsylvania, and Rhode Island. Laboratories should determine if they must comply with these states' regulations for performing tests on residents from these states and adhere to reporting requirements for *M. tuberculosis* complex, and in some states, reporting requirements for NTM.

IDEAL ALGORITHM/ALTERNATIVE ALGORITHMS FOR TESTING SPECIMENS FOR MYCOBACTERIAL DISEASES

The algorithms for testing specimens for mycobacteria are complicated since several tests and several appropriate algorithms may be used in the clinical laboratory. Ultimately, any methods and algorithms used should ensure that the *M. tuberculosis* complex is detected or ruled out as quickly as possible and that NTM are detected and identified to help to determine if they are clinically significant. This section provides a testing algorithm for specimens for TB and discusses some of the alternative options, including the decision to refer services to another laboratory.

Figure 1 shows an ideal diagnostic algorithm as proposed by the writing committee of this document for specimens from patients with suspected TB and specimens from patients with a low suspicion for TB but who need mycobacterial infection ruled out. Determining the best algorithm is dependent on the facility, its staffing and testing capabilities, and the patient population served. Some issues to consider when choosing an algorithm are the number of specimens that are tested, the prevalence of TB and other mycobacterial diseases in the patient population served (for example, a low-prevalence setting), the skills of the laboratory staff, the availability of appropriate facilities for handling and manipulating mycobacterial organisms, and the availability of reference or public health laboratory services (105).

Tests for Detecting or Ruling Out *Mycobacterium tuberculosis* Complex Infection

Any algorithm for testing specimens from patients with suspected TB should include a NAAT for the *M. tuberculosis* complex. The NAAT should ideally be performed on all first-time TB-suspect patients, and results should be reported within 48 h of specimen receipt (106). A NAAT should be performed directly on the specimen, and there are several methods available, including the commercial MTD and Xpert assays as well as laboratory-developed tests. NAATs are discussed in more detail in Nucleic Acid Amplification Tests, below.

NAATs may need to be modified and validated for off-label use for other specimen types with the caveat that test performance may be different. It is important to determine the best NAAT for the patient population served by the laboratory and whether NAATs will be performed on respiratory specimens only or whether nonrespiratory specimens will also be validated and tested. An additional consideration is whether a NAAT will be performed on at least one specimen from all individuals who are first-time TB-suspect patients or whether testing will include only AFB smear-positive specimens. For example, if the NAAT is performed on AFB smear-positive specimens only, then it will be performed on a concentrated, processed specimen after an AFB smear result is available. In the ideal algorithm, a NAAT is performed irrespective of the AFB smear result, and therefore, the specimen can be tested as soon as it is received, with results reported earlier. If NAAT for detection of TB is not offered by the laboratory, this test should be sent to a reference laboratory or to a state or local public health laboratory.

NAAT-positive specimens can be tested for resistance to at least RIF by a molecular method. A *M. tuberculosis* complex isolate that is resistant to RIF is often resistant to INH, and therefore, a molecular method that screens for resistance to at least RIF can be considered a proxy test for MDR-TB. In some cases, this screen may be performed

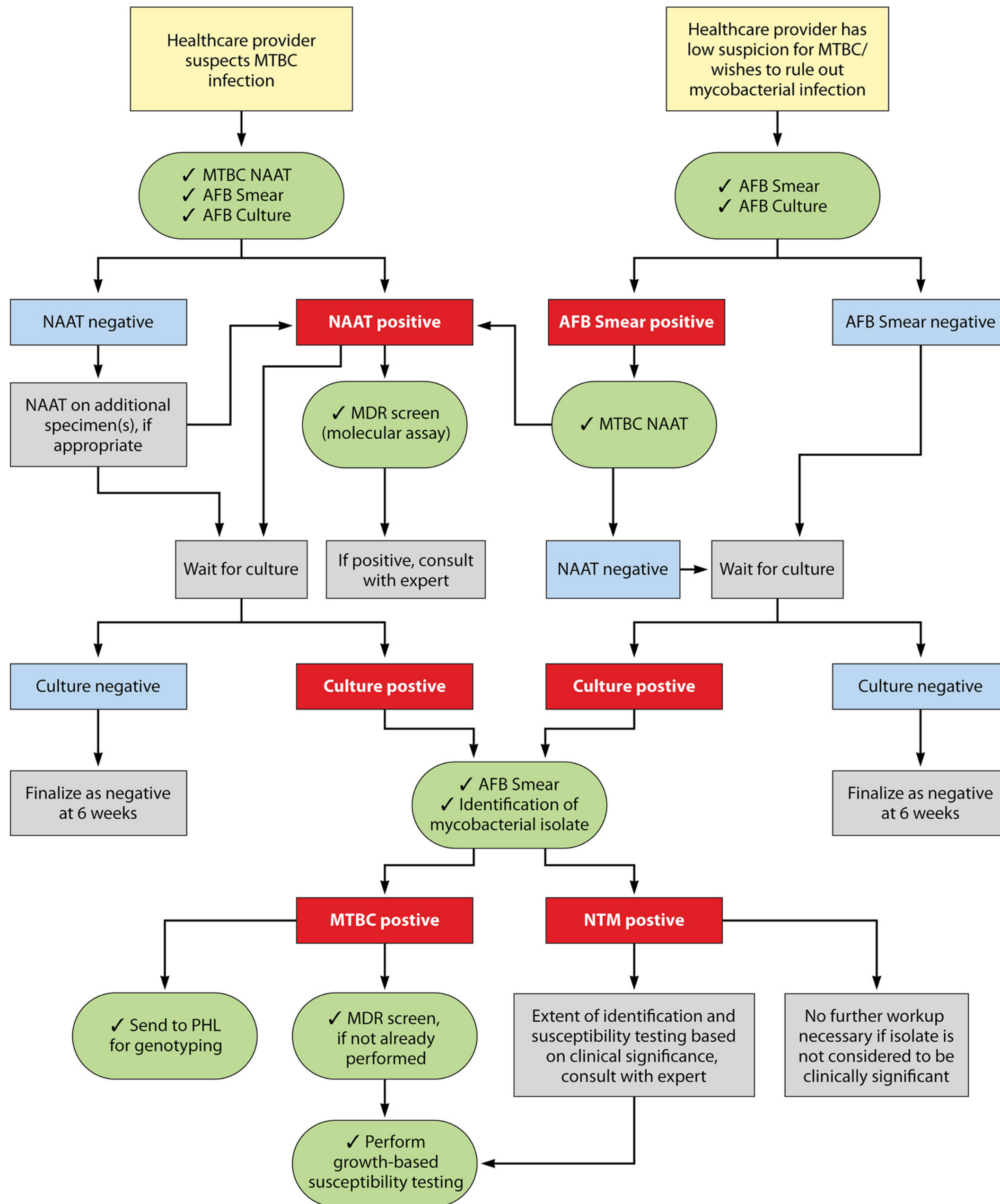

FIG 1 Schematic of the ideal algorithm for mycobacteriology testing in clinical and public health laboratories MTBC, *Mycobacterium tuberculosis* complex; NAAT, nucleic acid amplification test; AFB, acid-fast bacilli; MDR, multidrug resistant; PHL, public health laboratory.

only on patients with certain risk factors for MDR-TB, e.g., those who are foreign born, who have had contact with an MDR-TB case, who were previously treated for TB, or who were treated and were nonadherent. However, in the ideal algorithm, this screen is performed on all NAAT-positive specimens. Options for tests include the Xpert assay, which is an FDA-market-authorized test for the detection of the *M. tuberculosis* complex and mutations associated with RIF resistance. There are also several other laboratory-developed tests, such as Sanger sequencing and pyrosequencing, and research-use-only tests such as line probe assays, e.g., Hain Genotype MTBDR*plus* and MTBDR*sl*. With any laboratory-developed test, thorough validation must be performed prior to implementation to determine test performance and applicability (107–110). If the MDR screen, i.e., testing for molecular drug resistance, either RIF and INH or RIF only, is not

offered by the laboratory, the specimen should be sent to a reference laboratory or to a state or local public health laboratory. If the local or state public health laboratory does not have the capability to perform the MDR screen, specimens can be referred to the CDC, if certain criteria are met. The molecular detection of antimicrobial resistance using NAATs is discussed further in "Detection of Antimicrobial Resistance," below.

If the MDR-TB screen results are positive and RIF resistance (or RIF and INH resistance) is detected, further testing must be performed for confirmation and the detection of additional drug resistance. Additional testing allows the confirmation of RIF monoresistance versus MDR-TB and ruling out pre-XDR-TB or XDR-TB (111). These MDR-TB screen-positive specimens can be sent to a reference laboratory, to a state or local public health laboratory, or to the CDC through a U.S. public health laboratory.

Since 2012, the Molecular Detection of Drug Resistance (MDDR) program at the CDC has offered pyrosequencing and DNA sequencing of genes associated with resistance to RIF and INH to rapidly identify potential MDR-TB cases (see "Molecular AST methods," below). In addition, an extended panel that covers genetic loci associated with resistance to EMB, PZA, FQ, and the injectable agents (amikacin, kanamycin, and capreomycin) can be performed to detect XDR-TB cases. The MDDR program accepts isolates and NAAT-positive sputum sediments submitted by a U.S. public health laboratory. Submission must be preapproved by the CDC and is dependent on patient risk factors for MDR-TB and clinical history, including a known resistance result and drug allergy. The MDDR service can also provide molecular testing in situations where there are mixed/nonviable cultures and conventional methods are unsuccessful (112). NAATs for the detection of antimicrobial resistance are discussed further in "Detection of Antimicrobial Resistance," below.

An AFB smear and a culture should be performed on all specimens submitted for mycobacterial testing, except for blood, for which an AFB smear is not applicable. A concentration method and a fluorescent-stain technique that enhances the ability to identify AFB in a specimen should be used, as detailed in Smear Microscopy and Growth Detection of Acid-Fast Bacilli, below. When an AFB smear is performed to confirm the presence of AFB in a culture, a Ziehl-Neelsen or Kinyoun stain is adequate. It is essential that specimens be inoculated in solid and liquid media to increase the recovery of mycobacteria. In most cases, cultures can be finalized as negative at 6 weeks/42 days when using a commercial broth system. One alternative to this protocol would be to incubate cultures for an additional 2 weeks before reporting the culture as negative if, for example, the AFB smear is positive or a fastidious organism is suspected, e.g., *M. genavense* or *M. haemophilum*. If specialized media or growth conditions that are not available in the facility are required, the specimen should be sent to a reference laboratory. The details of these methods are discussed in Work-Up of Specimens, below. Culture is still the gold standard for the laboratory diagnosis of TB disease. Instrument-flagged broth cultures or growth on solid medium requires confirmation of AFB by smear microscopy prior to performing identification. Identification methods may include molecular methods such as Sanger sequencing, the use of DNA probes, NAATs, lateral flow assays, HPLC, or MALDI-TOF MS (details of these identification methods are discussed in Identification, below). Whichever methods are chosen, they should be performed frequently enough so that the *M. tuberculosis* complex can be identified or ruled out within 24 h of detection of an AFB-positive culture. Also, species identification of members of the *M. tuberculosis* complex (113) may need to be considered since treatment for *Mycobacterium bovis* BCG-*Mycobacterium bovis* differs from treatment for other *M. tuberculosis* complex species (12, 114). If a NTM is isolated, test methods should be available to provide species-level and subspecies-level identification for clinically significant isolates. Alternatively, these specimens may also be referred to another laboratory for identification, if warranted.

Once a culture has been confirmed to be positive for *M. tuberculosis* complex, AST should be performed by a culture-based method; however, there are a limited number of commercially available methods in the United States. The agar proportion (AP) method is the gold standard but requires considerable technical expertise. Details on

automated broth susceptibility systems can be found in Antimicrobial Susceptibility Testing, below. AST needs to be performed on at least one isolate from a first-time TB patient and can be repeated after 60 days if the patient is not improving clinically or has not converted to AFB smear-negative status. If a member of the *M. tuberculosis* complex is isolated from a different specimen source, it may also be appropriate to perform AST on a single isolate from that source. At least one isolate from each TB case should be sent to the jurisdictional public health laboratory for genotyping (details of the public health laboratory requirements can be found in Public Health Requirements, below).

Tests for Detecting or Ruling Out NTM/Low Suspicion for TB Disease

As with *M. tuberculosis* complex, any testing algorithm for the detection of NTM should always include an AFB smear and culture. See above for discussion regarding ideal testing for AFB smear and culture.

The first step in any testing algorithm for NTM should be a rapid method to rule out TB. In certain patient populations, such as CF patients or other immunocompromised patients, a rapid method to detect NTM may also be valuable. For example, some laboratories perform a laboratory-developed multiplex assay for the detection of the *M. tuberculosis* complex and one of the more common NTM, MAC. Otherwise, waiting for growth and performing identification of NTM isolates is the standard approach. Instrument-flagged broth cultures or growth on solid medium requires confirmation as AFB by smear microscopy prior to performing identification. As indicated above, there are various methods for the identification of mycobacteria. Methods such as HPLC and conventional biochemicals with phenotypic characteristics may be used, but some of these more traditional methods are not discriminative enough with the changing nomenclature and expanding number of new species within the genus *Mycobacterium* (details of these identification methods are discussed in Identification, below). If a NTM is identified, consultation with a physician experienced in NTM diseases may be necessary to determine whether the NTM is clinically significant and whether further testing, including AST, is warranted. The recovery and identification of NTM in CF and other immunocompromised patients are particularly important. For example, consideration of NTM pulmonary disease in CF patients should be based on clinical and laboratory findings. If NTM are identified, AST is essential to guide appropriate therapy (99). Finally, appropriate methods and technical skills should be available to identify mixed infections with *M. tuberculosis* complex-NTM or mixed species of NTM. If appropriate methods and technical skill are not available to work up mixed cultures, the culture should be sent to a reference laboratory or public health laboratory.

Algorithm Summary/Frequently Asked Questions

The following six questions were identified and answered by the writing committee of this document and are based on available guidelines and expert opinions.

Can NAAT replace AFB smear? Although NAAT is a rapid method for detecting TB, there can be false-negative results, depending on the quality of the specimen, the specific NAAT used, the bacterial load in the specimen, and sampling bias within specimens. In addition, most NAATs do not detect NTM, and a negative NAAT result for TB and a positive AFB smear are good indicators of the presence of NTM. In the algorithm, the NAAT and AFB smear can be performed concurrently so that the NAAT is not dependent on the result of the AFB smear. It is important for the health care provider to interpret the NAAT result in conjunction with that of the AFB smear.

Do an AFB smear and culture need to be in the algorithm when a NAAT is performed? AFB smear and culture should always be performed. An AFB-positive smear result gives the health care provider with valuable information. Culture is more sensitive and is still the gold standard.

When should a reflex MDR-TB screen be performed on a NAAT-positive specimen? A reflex MDR-TB screen should ideally be performed on all first NAAT-positive specimens or on initial TB isolates if the MDR-TB screen was not performed on the

original specimen or sediment. If this test is not performed, referring the specimen to a reference laboratory or a local or state public health laboratory should be considered.

When should identification be performed on a specimen positive for AFB? Identification should be performed on all AFB-positive specimens, as they may be clinically significant. It is important to rule *M. tuberculosis* complex in or out once a culture becomes AFB positive. Ideally, this testing should be performed daily. If a laboratory is performing culture, having probes for identification may be useful, as they can provide a fast result for the most common species. If identification methods are not available, laboratories should refer isolates to an appropriate reference laboratory or public health laboratory.

How does the algorithm change for nonrespiratory specimens? At this time, there is no FDA-approved or -cleared NAAT for nonrespiratory specimens. It is recommended that each laboratory determine the need for testing on these specimen types and the performance characteristics associated with such testing.

What should be done when discrepant test results are obtained? The introduction of NAATs to the diagnostic options can yield discrepant test results, where one method yields a positive test result and another method yields a negative test result. As the use of NAATs and other molecular methods increases, discrepancies among these test results will occur. Clinical interpretations of discrepant test results can be challenging. The explanations for these discrepancies include specimen quality, the fact that molecular tests may target different sections of the microbial genome, and sampling bias. Most importantly, it should be remembered that no single test is 100% accurate, and whenever multiple tests for the same analyte are performed, there may be discrepant results between tests.

Health care providers interpreting discrepant results may be referred to a TB expert for assistance. In the United States, health care providers may contact Tuberculosis Centers of Excellence (funded by the CDC), in addition to other TB experts (115).

BIOSAFETY

The most important biosafety issue for diagnostic testing of specimens that could contain mycobacteria is that of the *M. tuberculosis* complex and aerosolization of bacilli in droplet nuclei. Protection of workers from laboratory transmission requires the use of standard laboratory precautions, good laboratory practices, safety devices, personal protective equipment, and appropriate decontamination and disposal of biological hazards. The use of sharps should be carefully assessed, i.e., replacement of glass pipettes by plastic pipettes when possible and evaluation of how needles and syringes are used in the laboratory. Because there is no *a priori* method of knowing which specimens may contain mycobacteria and which do not, all steps in specimen acquisition, processing, and disposal must be designed to protect providers and laboratory personnel from exposure to infectious droplet nuclei (116).

Risk Assessment

Because the scope and scale of testing vary widely among laboratories, no single set of safety guidelines or recommendations is applicable to all laboratories that perform mycobacteriology testing. Every laboratory should perform risk assessment and stratification that can be used to guide the development of a biosafety program for that laboratory (117). Approaches to risk assessment and stratification are available from the WHO (118), the Association of Public Health Laboratories (103), the U.S. DHHS, and the CDC (119). Performing risk assessment and stratification allows laboratory directors to design risk mitigation programs appropriate for each laboratory. As detailed in the WHO document, laboratories can be divided into low-, moderate-, and high-risk categories (118). Low-risk laboratories, which typically manipulate specimens only to perform AFB smears with or without the use of a molecular assay, correlate with U.S. DHHS recommendations for using biosafety level 2 (BSL-2) conditions and practices. Moderate-risk laboratories, which process specimens for inoculation onto culture media and may perform direct AST (from a processed AFB smear-positive sediment), correlate with U.S.

DHHS recommendations for using BSL-3 conditions and practices (119). High-risk laboratories, which process mycobacterial cultures to identify *M. tuberculosis* and may perform indirect AST (from a positive TB culture), also correlate with CDC recommendations for using BSL-3 conditions and practices (119). As the use of molecular diagnostic assays increases, particularly self-contained systems with little or no need for specimen processing (e.g., Xpert), risk assessment and stratification may be necessary in parts of the laboratory that traditionally have not performed mycobacteriology testing.

Specimen Processing

All specimen processing must be performed in a biosafety cabinet (BSC), in a BSL-2 or BSL-3 laboratory. In addition, all steps involving cultures, including plating, examination of plates/slants, subcultures, and manipulation of plates/slants/tubes, must be performed in a BSC and, ideally, in a laboratory meeting BSL-3 requirements (117). Vials placed into automated instruments can be handled safely outside a BSC until there is a need to remove aliquots of the broth, such as for stains, subcultures, and molecular assays. The risk assessment may determine if flagged positive vials may need to be transported using a leakproof container from room to room. A common question is whether AFB smears can be stained outside a BSC; the broad answer is "yes," because once prepared and fixed, AFB smears do not aerosolize readily and thus pose little biological hazard to laboratory staff when stained outside a BSC. In addition, as noted by Chedore et al., chemical fixation with 5% phenol in ethanol is superior to heat fixation by passage through the blue cone of a flame of a gas burner (120). A risk assessment should be used to determine the best way to prepare and fix AFB smears for one's laboratory. In the same way, examination of sealed tubes containing liquid media, sealed slants, sealed petri dishes, or petri dishes contained within sealed plastic bags can be done safely at the bench so long as BSL-2 conditions are met and a risk assessment has been conducted.

Several aspects particular to the Xpert assay suggest that it might present a very low risk to testing personnel (121). The Xpert assay starts with the addition of a highly tuberculocidal sample treatment reagent to each sputum sample (or concentrated sputum pellet). Used as recommended, the use of sample treatment reagent complies with international decontamination standards (122, 123), reducing the viability of *M. tuberculosis* bacteria in sputum by at least 6 $\log_{10}$ units after 15 min of incubation (124). Longer incubation periods appear to completely sterilize samples spiked with very high numbers of *M. tuberculosis* bacteria. After a 15-min incubation, the sputum sample treatment reagent mixture is transferred to a plastic assay cartridge, the cartridge lid is closed, and the cartridge is placed into an Xpert instrument. The remainder of the assay is performed within the closed cartridge. The use of a closed-cartridge system can potentially further reduce or eliminate the biohazard risk by performing sample processing in an aerosol-resistant enclosure. The biosafety precautions for performing the Xpert assay are equivalent to the requirements for performing direct sputum AFB smear microscopy, i.e., minimal (125, 126).

Decontamination and Disposal of Laboratory Waste

The 2012 CDC guidelines for safe work practices for human and animal medical diagnostic laboratories (117) state the following regarding mycobacteriology laboratory waste. (i) Provide an autoclave in the mycobacteriology laboratory so that generated waste can be sterilized before transport from the laboratory. Adhere to the scheduled quality control and maintenance procedures for the autoclave. (ii) If an autoclave is not available or for items that cannot be autoclaved, all waste from the mycobacteriology laboratory must be securely contained in leakproof containers. Package waste so that the outside of the container can be disinfected before it leaves the laboratory. (iii) Chemically disinfect waste materials before removal from the BSC.

The decision as to how to handle waste is part of the risk assessment for the laboratory and will also be driven by the type of facility and the facility's approach to

biohazard waste disposal (i.e., some facilities still autoclave all biohazard waste on site; others do not). It bears emphasizing that some types of biological waste cannot be autoclaved safely, the most common examples being waste containing bleach or phenolic compounds.

Disposal of all laboratory waste requires compliance not only with safety guidelines but also with federal regulations for protecting patient confidentiality, a task achieved primarily through compliance with the Health Insurance Portability and Accountability Act (HIPAA). Disposal of laboratory and other hospital/clinic waste poses unique challenges due to the presence of many types of labels attached to a wide variety of products. Typically, the removal of labels manually is not an option due to the time and effort required as well as the observation that parts of labels often remain attached to products. The most common approach is to use labels that darken during autoclaving, thereby obscuring the wording and numbering on labels. Several commercial vendors manufacture such heat-sensitive labels, but it is strongly recommended that each laboratory verify that these labels work as intended under local conditions.

WORK-UP OF SPECIMENS

This section addresses various aspects of the laboratory diagnosis of mycobacterial infections: acceptable specimens and rejection criteria, specimen processing, and media. The intent of this section is not to delineate specific protocols for processing but rather to provide overall principles regarding various aspects of laboratory diagnosis as well as other details to aid in protocol development.

Acceptable Specimens and Rejection Criteria

Most specimens sent to the laboratory that are acceptable for routine bacterial culture are also acceptable for processing for AFB; however, every laboratory should develop specific criteria for acceptance and rejection (1, 103, 115) to provide methods for the optimal isolation of *Mycobacterium* spp. Most specimens will be obtained from the respiratory tract, especially expectorated and induced sputum (the optimal volume is 5 to 10 ml), bronchial aspirates, and bronchoalveolar lavage fluids. Furthermore, the 2017 ATS/IDSA/CDC TB diagnosis guidelines recommend that postbronchoscopy sputum specimens be collected from all adults with suspected pulmonary TB who undergo bronchoscopy (98).

A systematic review by Ho et al. (127) found that microscopic sputum quality assessment and rejection criteria used for typical bacterial culture are not relevant to the assessment of sputum quality for AFB smear and culture. Designed to detect sputum contaminated with upper respiratory flora, the two criteria commonly used to assess sputum quality for bacterial culture, i.e., low numbers of sputum squamous epithelial cells and white blood cell/squamous epithelial cell ratios, were poor predictors of *M. tuberculosis*-positive sputum. However, since the sputum decontamination process used before the inoculation of media for mycobacteria eliminates most contaminating organisms, the rejection of contaminated specimens by using these parameters confers no specific advantage.

Khan et al. (128) found that, despite being the least technical and time-consuming method, visual assessment to determine whether the specimen is sputum or saliva rejected the lowest proportion of AFB-positive specimens (0.3%). Most microscopic grading criteria, particularly those that considered the squamous epithelial cell count, rejected a large proportion of specimens (30 to 66%), a sizeable fraction of which contained AFB (6 to 12%).

Also acceptable for processing for AFB are body fluids such as urine; peritoneal, pleural, synovial, and pericardial fluids; and tissue biopsy specimens of lung, liver, and kidney, etc. Fluids, tissues, and lavage fluids (gastric and bronchoalveolar) are much better for AFB recovery than are specimens collected on swabs; swab specimens should be discouraged. Since the sensitivity of stool AFB smears is only 32 to 34%, AFB smear results should not determine whether a culture for mycobacteria is performed (129). The use of stool specimens from patients other than persons living with HIV should be

discouraged. Cerebrospinal fluid (CSF) is an acceptable specimen for mycobacterial processing provided that an adequate volume is obtained (5 to 10 ml is preferred). However, the incidence of mycobacterial meningitis is low, especially in high-income countries, and CSF culture for mycobacteria should be performed when the results of CSF chemistry tests and the CSF cell count and differential indicate possible infection with mycobacteria, i.e., high protein levels, low glucose levels, and the presence of lymphocytes (130, 131).

Processing of inappropriate clinical specimens for mycobacterial detection is a waste of both financial and personnel resources. Discussion with clinicians and other health professionals may be needed to explain why the rejection criteria need to be enforced. These criteria should be readily available online and in procedure manuals.

Before discarding any patient specimens, it is prudent to discuss the circumstances of suboptimal specimen collection for mycobacteria with the health care provider. In cases where the specimen is less common, involves an invasive procedure, or could not otherwise be easily re-collected, an exception to the rejection criteria may be justified. In addition to general criteria for specimen rejection (e.g., unlabeled or mislabeled specimens), rejection criteria for AFB smear and culture should include the following (1, 132). (i) There are inadequate amounts of a specimen, e.g., $<$3 ml of sputum. (ii) The specimens are watery expectorated sputum that contains predominantly saliva (note that induced sputum may resemble saliva, and therefore, "induced sputum" should be specifically identified on the requisition form). (iii) Dry swabs (along with swabs in general) should be discouraged; however, the ESwab may be suitable for preserving the viability of *Mycobacterium* species (133). Preferred specimens for AFB recovery should be tissues and body fluids; swabs are not recommended for the isolation of mycobacteria, since they provide limited material. Swabs may be acceptable only if a specimen cannot be collected by other means, and a disclaimer should be included in the report. (iv) Samples are old, usually considered to be $>$7 days from the date of collection (132). (v) Sputum or urine is collected as a pooled specimen. (vi) The specimen is a formalin-fixed tissue specimen for growth detection; however, it may be suitable for molecular detection, especially when it is the only specimen type available (for detailed information regarding collection and transport, storage conditions for specimens prior to testing, as well as minimum volumes, see reference 132).

Processing of Specimens for Mycobacterial Stains and Culture

Due to the lengthy incubation times required for the growth of most *Mycobacterium* spp., methods to eliminate non-AFB and fungi should be employed before plating of the specimens. Sterile tissues and body fluids may not need decontamination; however, sputum, bronchoalveolar lavage fluid, stool, urine, skin, and many other specimens that usually contain contaminating microbes need to be decontaminated to allow the growth of mycobacteria. The chosen decontamination process should not seriously affect the viability of mycobacteria. All samples, sterile and nonsterile, should also be concentrated to maximize the recovery of mycobacteria. For viscous specimens such as sputum, liquefaction is necessary, along with decontamination and concentration.

In its manual (134), the WHO Global Laboratory Initiative recommends the inclusion of one negative-control specimen (to control for cross-contamination) and one positive-control specimen (H37Rv or H37Ra, to verify the accuracy of the methods) weekly with the processing of a batch of clinical specimens. These control specimens must be included near the end of the batch and handled as patient specimens being processed for AFB smear microscopy and culture. However, the inclusion of a positive control in the processing of patient specimens is controversial and is discouraged in the United States due to the risk of cross-contamination (1).

Sterile specimens. Tissues can be ground with 0.85% sterile saline or 0.2% sterile bovine albumin and then inoculated into broth and solid media. Body fluids, e.g., CSF, pleural effusions, or ascites, should first be concentrated by centrifugation at $\geq$3,000 $\times$ *g* for 15 min prior to the inoculation of the resultant sediment. However, if the volume of body fluid submitted for culture is small and cannot be obtained again, i.e.,

TABLE 5 Digestion and decontamination methods for processing of AFB specimens[a]

Decontaminant(s) (reference)	Indication(s)
NALC–2% NaOH (75)	Decontamination of sputum and other nonsterile specimen types
Oxalic acid (75)	Specimens known to be contaminated with *Pseudomonas* spp.; CF patients
NALC-NaOH and oxalic acid (2-step procedure) (137)	Respiratory specimens from CF patients
Chlorhexidine (138)	Respiratory specimens from children and adolescents with CF and from CF patients of any age with suspected *M. abscessus* infections (may not be compatible with the MGIT detection system)
Sulfuric acid (75)	Urine that consistently yields a contaminated culture when processed with NALC-NaOH

[a]NALC, *N*-acetyl-L-cysteine; NaOH, sodium hydroxide; CF, cystic fibrosis.

irreplaceable, it may be added directly to liquid media (1). Sterile specimens should be transported to and processed in the laboratory as soon as possible. If transportation to the laboratory is delayed by more than 1 h from the time of collection, with the exception of blood and CSF, specimens should be refrigerated at 2°C to 8°C (135).

Blood collection tubes should contain sodium polyanethol sulfonate (SPS), heparin, or citrate anticoagulants and be transported expeditiously to the laboratory. EDTA tubes should not be used (1).

Nonsterile or contaminated specimens. Liquefaction of the specimen and decontamination are essential for sputum and other respiratory tract specimens, stool, skin scrapings, and urine. It is important to mix the solution vigorously until it is liquefied (about 5 to 20 s on a test tube mixer) and to invert each tube to ensure that the solution contacts all inside surfaces of the tube and cap. One of the most common decontaminants is sodium hydroxide (NaOH), which also serves as a mucolytic agent; however, NaOH can also be detrimental to mycobacteria unless it is used with caution regarding the concentration and time of exposure. More commonly, laboratories use a combination of liquefaction and decontamination using *N*-acetyl-L-cysteine (NALC) to liquefy the specimen without harming the bacterial cells, in conjunction with NaOH at a lower concentration than would be used with NaOH alone. The most commonly used combination is NALC–2% NaOH. Concentrations of up to 5 to 6% NaOH can be utilized for heavily contaminated specimens, although up to 90% of the mycobacteria may also be harmed (1). There are other decontamination agents used for specific specimen types, e.g., urine, and/or known patient diseases, e.g., CF (see Table 5 for a summary of the various decontaminating agents).

Nonsterile specimens should be transported to the laboratory and processed as soon as possible. If transportation to the laboratory is delayed by more than 1 h from the time of collection, specimens should be refrigerated at 2°C to 8°C (135).

There are some special considerations for selected specimens. Gastric lavage fluid should be processed within 4 h of collection or neutralized with 10% sodium bicarbonate and then refrigerated if further processing is delayed (75). Urine can be distributed in up to four 50-ml conical tubes, and the sediments can be combined; up to 1 g of formed stool samples should be emulsified in saline and thoroughly vortexed, and the particles should be allowed to settle out before processing the top portion of the liquid (1).

Attempting to recover NTM from a respiratory specimen obtained from a CF patient is challenging for the laboratory (136). Approximately 50% to 70% of respiratory specimens from CF patients contain *Pseudomonas aeruginosa*, which is resistant to NALC-NaOH decontamination; thus, after decontamination with NALC-NaOH, specimen contamination rates of between 35% and 70% occur. When a second step using 2.5% oxalic acid is added, the contamination rate is reduced to 3% to 5%, although low numbers of MAC bacteria may not be recovered (137). Chlorhexidine alone has been shown to be an effective means of decontaminating CF respiratory specimens by using solid media (138). As an added value, pretreatment of sputum from CF patients with chlorhexidine yielded twice as many NTM-positive cultures as specimens treated with NALC-NaOH (136). Chlorhexidine, which is potentially toxic to mycobacteria, must be inactivated by incorporation with egg lecithin in the culture medium. Asmar et al. (139)

TABLE 6 Various types of media used in the mycobacteriology laboratory[a]

Medium[b]	Description
Solid egg based (supports good growth of most mycobacteria)	
LJ	Good for *M. tuberculosis* complex; inferior for NTM recovery; although CO_2 is not essential to initiate growth, it stimulates earlier and more luxuriant growth
Gruft modification of LJ	Contains antibiotics (penicillin and nalidixic acid) that may be useful to control excessive contamination; although CO_2 is not essential to initiate growth, it stimulates earlier and more luxuriant growth
Agar based (less contamination due to more simple formulation and thus less likely to support growth of contaminants)	
Middlebrook 7H10	Transparent, so colonies can be more easily observed; requires incubation with 5 to 10% CO_2 for maximum yield
Middlebrook 7H11	7H10 agar enriched with casein digest; transparent, so colonies can be more easily observed; requires incubation with 5 to 10% CO_2 for maximum yield; stimulates more luxuriant growth of drug-resistant *M. tuberculosis* complex bacteria
Mitchison's selective 7H10 and 7H11	Contain the antibiotics carbenicillin, polymyxin B, trimethoprim lactate, and amphotericin B; suppressive for most contaminants; McClatchy et al. found higher yield of mycobacteria on selective 7H11 medium than on nonselective 7H11 medium[c]; can be used in conjunction with nonselective media to increase culture yields
TLA system	Thinly poured plates of Middlebrook 7H11 agar; microcolonies can be detected and work-up can be started earlier than with traditional solid media; this method can be labor-intensive, but for laboratories that cannot afford automated broth detection systems, the time to detection with TLA is equivalent, without the expense of an instrument

[a]See references 75 and 335.
[b]LJ, Löwenstein-Jensen; TLA, thin-layer agar.
[c]See reference 336.

demonstrated the superiority of a chlorhexidine decontamination procedure combined with a new agar medium, MOD9, compared to NALC-NaOH and MGIT960 for the isolation of *M. tuberculosis*.

Media

A combination of broth and solid media should be included for inoculation. Broth medium is essential for the recovery of *Mycobacterium* spp. in as short an incubation time as possible. There are many types of broth media, many of which are included with automated mycobacterial detection systems; most are based on Middlebrook 7H9 broth. Solid media (egg based and non-egg based) are included to ensure the recovery of rare strains that may not grow in broth. Table 6 lists the various types of media that are available, while Table 7 summarizes salient features of automated broth-based culture systems. Of significance, some decontamination/concentration procedures are known to be compatible with egg-based media only and may not be used with any other media not containing egg yolk. These procedures include the use of Zephiran-trisodium phosphate (Z-TSP), sodium lauryl sulfate, cetylpyridinium chloride, or other quaternary ammonium compounds (140).

Tubed media should be incubated in a slanted position with screw caps loose for at least 1 week to ensure an even distribution of the inoculum (75). Thereafter, if space is needed, tubes may be placed upright with the caps tightened to minimize evaporation and drying of media. Plated medium, ideally placed into CO_2-permeable plastic bags or sealed with CO_2-permeable tape, should be incubated with the medium side down if all the inoculum has not been absorbed (75).

For the isolation of mycobacteria, the use of a liquid medium in combination with at least one solid medium is essential for good laboratory practice (141). Broth medium allows the more rapid growth and detection of mycobacteria and, therefore, the more rapid detection of the *M. tuberculosis* complex than does solid medium.

Specifically with respect to cultures of respiratory specimens from CF patients, it was recently reported that *M. abscessus* and its subspecies as well as other rapidly growing mycobacteria can be recovered on a medium that is routinely used for the isolation of

TABLE 7 Continuously monitored broth culture instrument systems[a]

System(s)[b]	Description[c]	Advantage and limitation(s) (reference)
BD Bactec MGIT automated mycobacterial systems (960 and 320 systems, depending on vol; BD Diagnostic Systems, Sparks, MD)	System automatically detects tube placement, i.e., scan-and-load technology; uses MGIT medium containing patented sensors that detect oxygen consumption, indicating mycobacterial growth; PANTA antibiotic mixture is added to the MGIT tube prior to inoculation; a fluorescent compound, embedded in silicone in the tube bottom, is quenched by the presence of oxygen, but when the oxygen is metabolized by mycobacteria or another microorganism, the compound fluoresces	Time to detection is much shorter with this system than with solid media; mycobacterial growth, including *M. tuberculosis* complex, *M. xenopi*, and various other NTM, can go undetected by the instrument (337); tubes should be visually inspected for clumps before the tubes are discharged as negative (338)
BacT/Alert Mycobacteria detection system (bioMérieux, Durham, NC)	Utilizes a colorimetric sensor and reflected light to monitor the presence and production of CO_2; the sensor changes color because of CO_2 produced by the metabolic activity of mycobacteria and other microorganisms; enrichment fluid containing bovine serum albumin, sodium chloride, oleic acid, and saponin is added; bottles contain antibiotic mixture of amphotericin B, azlocillin, nalidixic acid, polymyxin B, trimethoprim, and additional growth factors	Time to detection is much shorter with this system than with solid media; mycobacterial growth can go undetected by the instrument; tubes should be visually inspected for clumps before the tubes are discarded as negative; negative cultures may also be checked by AFB smear and/or subcultured at some point prior to discarding the specimen as negative (339)
VersaTREK (ThermoFisher Scientific, Waltham, MA)	Bottles contain antibiotic mixture of polymyxin B, vancomycin, nalidixic acid, and amphotericin B; each bottle is continuously monitored for changes in gas pressure, due to consumption of oxygen by mycobacteria and production of gas by their metabolic activity	Time to detection is much shorter with this system than with solid media; at the end of the incubation period, a bottle not exhibiting a positive growth response should be visually inspected for turbidity (340)

[a]Mycobacterial blood culture methods in common use include visual inspection of processed blood (e.g., Isolator-10 system; Inverness Medical, Princeton, NJ) inoculated on solid medium, continuous detection in liquid medium inoculated with blood (e.g., BacT/Alert MB system or Bactec Myco/F Lytic system) (341), or processed blood (e.g., VersaTREK) (340). The turnaround times to detection for *M. tuberculosis* were 23.8 days for the Isolator-10 system, 20.0 days for the Myco/F Lytic system, and 16.5 days for BacT/Alert MB (341).

[b]See reference 1.

[c]PANTA, polymyxin B, amphotericin, nalidixic acid, trimethoprim, and azlocillin.

bacteria from CF patients, *Burkholderia cepacia* selective agar (BCSA) (142, 143). *M. abscessus* clinical isolates were recovered on BCSA from 65% to 75% of infected individuals, whereas these organisms were recovered from 85% of patients by using mycobacterium-specific culture methods. In addition, incubation of BCSA plates for 14 days greatly enhanced recovery compared with a 5-day incubation (142). More recently, a newly developed medium, namely, RGM, was introduced for the isolation of rapidly growing mycobacteria from the sputum of CF patients without the need for decontamination of specimens (143–146). RGM supported the growth of all mycobacteria tested and was more selective than BCSA (144).

NUCLEIC ACID AMPLIFICATION TESTS

Diagnostic

Algorithms for testing specimens from patients suspected of having TB should include a NAAT. Guidelines from the CDC recommend the performance of a TB NAAT on at least one respiratory specimen from patients with signs and symptoms consistent with pulmonary TB. This is especially important if a diagnosis of TB has not yet been made and the test result would alter patient care and/or TB control activities (106). Based on CDC guidelines, an institution may want to develop criteria to identify patients suspected of having TB. Although the preference is to test the first collected specimen by a NAAT to decrease the time to a result, an AFB smear-positive specimen should take priority due to the increased sensitivity of NAATs for these specimens. It is important to note that NAATs should not replace or delay routine microbiological methods, including AFB smear. The NAAT should ideally be performed on all first-time

TABLE 8 Characteristics of FDA-cleared and FDA-market-authorized NAATs for *M. tuberculosis* complex detection according to the manufacturers' package inserts[b]

Test	Specimen types	Methodology	Sensitivities (%)		Specificity (%)		Tests for inhibitors
			AFB smear negative	AFB smear positive	AFB smear negative	AFB smear positive	
Hologic Amplified MTD	AFB smear-positive and -negative concentrated sediments prepared from sputum, bronchial specimens, or tracheal aspirates	Transcription-mediated amplification of rRNA and detection by hybridization protection assay	64.0 for single specimen, 71.4 for 2 specimens	87.5 for single specimen, 100 for 2 specimens	99.1–100	100	No[a]
Cepheid Xpert MTB/RIF	AFB smear-positive and smear-negative sputum samples or concentrated sediments prepared from sputum	Real-time PCR using molecular beacon probes to the 81-bp rifampin resistance-determining region of *rpoB*	73.1 for single specimen, 90.0 for 3 specimens	97.8 for single specimen, 99.5 for 3 specimens	97.9–99.0	97.9–99.0	Yes

[a]Can be assessed with additional steps.
[b]See references 147 and 148. AFB, acid-fast bacilli.

patients with suspected TB, and results should be reported within 48 h of specimen receipt (106).

There are two commercial NAATs for the detection of *M. tuberculosis* complex DNA directly from respiratory specimens: the MTD test and the FDA-market-authorized Xpert test. There are also many laboratory-developed NAATs being used in clinical and public health laboratories. Xpert is authorized for use with AFB smear-positive and AFB smear-negative expectorated and induced raw sputum specimens as well as processed sputum sediment, and the MTD test is approved for both AFB smear-positive and AFB smear-negative respiratory specimens (147, 148). Although the specificity of NAATs for TB diagnosis is very high, the sensitivities vary widely and depend on both the AFB smear status of the specimen (positive or negative) and the specimen type (147, 148). Table 8 summarizes the claims and performance characteristics of these two commercial assays.

NAATs may need to be modified and validated for off-label use for other specimen types with the caveat that the test performance may be different. It is important to determine the best NAAT for the patient population served by the laboratory and whether NAATs will be performed on respiratory specimens only or whether nonrespiratory specimens will also be validated and tested. An additional consideration is whether NAATs will be performed on at least one specimen from individuals who are first-time TB-suspect patients or whether testing will include only AFB smear-positive specimens. For example, if the NAAT is performed on AFB smear-positive specimens only, it will be performed on a concentrated, processed specimen after an AFB smear result is available. In the ideal algorithm, a NAAT is performed irrespective of the AFB smear result, and therefore, the specimen can be tested as soon as it is received, with results being available sooner. If a NAAT is not offered by the laboratory, the specimen should be sent to a reference laboratory or to a state or local public health laboratory (see Table 9 for recommendations for interpretation of NAAT results).

Disadvantages of the molecular detection of the *M. tuberculosis* complex directly from clinical specimens include the inability to differentiate among species within the *M. tuberculosis* complex. In addition, a positive NAAT result does not differentiate between live and dead organisms. For this reason, amplification technologies should not be used on specimens collected from patients who have received antituberculosis drugs for more than 7 days or have received such therapy in the last 12 months prior to collection (148, 149). Furthermore, approximately 4% of pulmonary and 19% of extrapulmonary specimens have substances that are inhibitory to amplification (deter-

TABLE 9 CDC recommendations for interpretation of results of tuberculosis nucleic acid amplification tests on respiratory specimens[a]

AFB smear result	TB NAAT result	Interpretation
Positive	Positive	Presumed pulmonary TB Begin anti-TB therapy while awaiting culture and susceptibility results
Negative	Positive	Consider performing NAAT on a second specimen to confirm results Consistent with pulmonary TB but less infectious than AFB smear-positive TB Use clinical judgment regarding beginning anti-TB therapy
Positive	Negative	Rule out inhibition by using an internal amplification control Test additional specimen(s) by NAAT Consistent with the presence of NTM if all NAAT results are negative Use clinical judgment regarding beginning anti-TB therapy
Negative	Negative	Rule out inhibition by using an internal amplification control Multiple negative AFB smears and multiple negative NAAT results, in combination with other requirements, support discontinuation of airborne isolation Use clinical judgment regarding beginning anti-TB therapy

[a]See references 105, 106, and 342.

mined by a failed internal amplification control), making negative test results invalid (149). Since it is rare that all specimens from a given patient show inhibition, testing of multiple samples can be advantageous (150).

Detection of Antimicrobial Resistance

Due to the slow growth of the *M. tuberculosis* complex, molecular techniques are well suited not only to detect the *M. tuberculosis* complex directly from patient specimens but also to screen for mutations associated with antimicrobial resistance. Although Xpert is the only FDA-cleared product for the detection of RIF drug resistance in TB isolates in the United States, several assays have received a CE mark for clinical use in Europe. These assays include the Genotype MTBDR*plus* Version 2 system (Hain Lifescience, Germany) to detect RIF and INH resistance; the Autoimmun Diagnostika GmbH (Strasberg, Germany) TB Isoniazid, Rifampin assay; and the Innogenetics (Ghent, Belgium) INNO-LiPA Rif.TB assay. All these systems detect RIF resistance, since a main cause for treatment failure and fatal clinical outcome in TB patients is resistance to rifampin (346). In addition, RIF resistance can be a marker for MDR-TB in the appropriate setting (151).

RIF has long been used in combination first-line therapy for TB. The cellular target of RIF is the beta-subunit of bacterial DNA-dependent RNA polymerase, which is encoded by the *rpoB* gene. Point mutations in *rpoB* can render the organism resistant to RIF due to decreased binding affinity and can result in high-level resistance (152). RIF resistance is detected by analyzing the *rpoB* gene for specific mutations in the 81-bp RIF resistance-determining region (RRDR). The Xpert assay includes five probes that hybridize to the RRDR of the *M. tuberculosis* complex. RIF resistance is reported if at least one probe does not produce a signal (indicating a mutation at the hybridization site preventing hybridization) or if there is a $\geq$3.5-cycle difference between probe signals (indicating less efficient hybridization due to mutation) (147). Compared to the use of three sputum cultures per patient, the Xpert assay performed with 96.1% sensitivity and 98.6% specificity during a clinical trial (147, 151). However, the sites in the clinical trial (Peru, Azerbaijan, South Africa, and India) have a higher prevalence of TB and MDR-TB than the United States. Therefore, the positive predictive value is likely to be lower in the United States. Of note, a positive RIF result by Xpert can be obtained for synonymous (i.e., silent) mutations. For these reasons, it is critical that initial RIF resistance results be confirmed by traditional (i.e., phenotypic) AST. Additional molecular confirmation of results can also be rapidly obtained through the CDC's MDDR service (153). A summary of the CDC's recommendations for the interpretation of RIF resistance molecular assay results obtained by the Xpert test is presented in Table 10, but these recommendations also apply to other molecular detection assays as well.

TABLE 10 CDC recommendations for interpretation and reporting of positive Xpert results[a]

Xpert result	Interpretation	Suggested minimum reporting language
M. tuberculosis complex detected	*M. tuberculosis* complex DNA is detected in the sample	*M. tuberculosis* complex detected
Rifampin resistance detected	Mutation(s) is detected in *rpoB*	A mutation in *rpoB* has been detected, indicating possible rifampin resistance; confirmatory testing should follow
M. tuberculosis complex detected	*M. tuberculosis* complex DNA is detected in the sample	*M. tuberculosis* complex detected
Rifampin resistance not detected	Mutation(s) is not detected in *rpoB*	No *rpoB* mutations detected; probably rifampin susceptible
M. tuberculosis complex detected	*M. tuberculosis* complex DNA is detected in the sample	*M. tuberculosis* complex detected
Rifampin resistance indeterminate	Mutation(s) in *rpoB* could not be determined due to insufficient signal	Presence of *rpoB* mutations cannot be accurately determined

[a]Adapted from reference 342.

For the molecular detection of high-level INH resistance, the *katG* gene (coding for the catalase peroxidase) is examined, and for the detection of low-level INH resistance, the promoter region of the *inhA* gene (coding for the NAD [NADH] enoyl-acyl carrier protein reductase) can be analyzed. The recognition of these two loci is of clinical significance. In the presence of *inhA* mutations, INH resistance can usually be successfully controlled with high-dose INH therapy (154). The presence of an *inhA* mutation may also alert the clinician to not include ethionamide or prothionamide in the MDR-TB treatment regimen due to potential cross-resistance to these agents (115). The assumption that RIF-susceptible cases tested by the Xpert assay would also be INH susceptible is not necessarily true. In a study from Mumbai, India, by Vadwai et al. (155), 8.7% of TB patients tested by the Xpert assay were negative for RIF resistance; however, they demonstrated INH monoresistance phenotypically. This significant number of patients with INH-resistant TB, if remaining unidentified, will be treated with only a single active drug during the continuation phase of their therapy, which will inevitably lead to MDR-TB. These few points highlight the importance of molecular INH resistance screening (156).

Although several reports have described the molecular detection of RIF and/or INH resistance utilizing line probe, real-time PCR, or sequencing methodologies, it should be noted that conventional AST is still the reference method due to incomplete data on all mutations and/or target genes associated with phenotypic resistance. Therefore, the most appropriate use for the molecular detection of antimicrobial resistance is as an initial screening assay. This approach could potentially identify resistant isolates weeks earlier, allowing clinical care to be significantly improved.

Infection Control

In February 2015, the FDA cleared the Xpert assay to aid physicians in determining if patients with suspected TB can be removed from airborne infection isolation (AII) (157, 158). In a consensus statement by the U.S. National TB Controllers Association and the U.S. Association of Public Health Laboratories, it is noted that the process described here is not to be used alone to rule out TB; Xpert test-negative or AFB smear-negative sputum may contain viable organisms and represent infectious TB (160). Based on an international clinical trial (multiple U.S. sites, South Africa, and Brazil), one or two negative tests performed on respiratory specimens from patients with signs and symptoms of pulmonary TB may allow the patient to be removed from AII (159). This study included 219 subjects with culture-confirmed *M. tuberculosis* complex infection out of 992 total subjects (22%), 45% of whom were persons living with HIV. The performance of a single Xpert test detected ∼97% of patients who were AFB smear positive and had culture-confirmed *M. tuberculosis* complex infection, while the performance of two Xpert tests detected 100% of these patients. The use of one or two Xpert tests detected 55% or 69%, respectively, of sputum specimens from AFB smear-negative but culture-positive patients. Prior to the clearance of this indication by the FDA, removal from AII relied primarily on AFB smear results from three specimens

obtained at least 8 h apart. Studies have shown that the use of the Xpert test allowed the release of patients from AII earlier and can result in health care cost savings (161, 162). The decision of whether to use one or two Xpert tests to remove patients from AII should be based on clinical circumstances, institutional guidelines, and state requirements. Clinical decisions regarding the need for continued AII should be made in conjunction with other clinical and laboratory data and not based solely on negative Xpert results (158).

SMEAR MICROSCOPY AND GROWTH DETECTION OF ACID-FAST BACILLI

Because of their unusual outer cell wall structure that contains mycolic residues of peptidoglycolipids, mycobacteria resist decolorization by acidified alcohol after the application of certain arylmethane dyes such as basic fuchsin, in which stable complexes are formed between the mycobacteria and dye (163). Thus, mycobacteria can be distinguished from other bacteria by AFB smear microscopy. This key property of the mycobacterial cell wall is referred to as acid fastness.

Diagnostic AFB Smear and Growth Detection

Despite major advances in the diagnosis of mycobacterial infections, microscopic examination for AFB remains a primary tool because it identifies highly infectious AFB smear-positive cases, is rapid, and is inexpensive; its ease of preparation and availability make it particularly attractive in most global settings (specific aspects regarding AFB smear microscopy in middle- and low-resource settings are addressed in Resource-Limited Settings, below). Although it has been reported to have more than 80% sensitivity compared with culture for identifying cases of pulmonary TB in some settings, the sensitivity has also been reported to be lower and variable in other settings (164); of note, AFB smear microscopy is of limited utility for paucibacillary disease such as in pediatric patients, persons living with HIV, and those with extrapulmonary infections. For the initial diagnosis of pulmonary TB, one should collect a series of three sputum specimens, 8 to 24 h apart, at least one of which is an early-morning specimen (105).

The optimum number of sputum specimens to establish a diagnosis has been examined in several studies that have served to support recommendations to decrease the minimum number of sputum specimens examined from 3 to 2, assuming that they are examined in a quality-assured laboratory. In a systematic review of 37 studies on the yield of sputum AFB smear microscopy (165), it was found that, on average, the initial specimen was positive in 85.8% of all patients ultimately found to have AFB detected, the second specimen was positive in an additional 11.9% of patients, and the third specimen was positive in a further 2.3% of patients. In studies that used culture as the reference standard, the mean incremental yield in sensitivity of the second specimen was 11.1%, and that of the third specimen was 3.1%. Thus, it appears that in a diagnostic evaluation for TB, at least two specimens should be obtained. In some settings, because of practicality and logistics, a third specimen may be useful, but examination of more than two specimens adds minimally to the number of positive specimens obtained.

AFB Smear and Growth Detection for Patient Management

Sputum specimens for AFB smear and culture should be obtained monthly until two consecutive specimens are negative upon culture (166). Collection of sputum specimens more often early in treatment for assessment of the treatment response and at the end of treatment is optional. If the patient remains culture positive after completing 3 months of treatment, AST should be repeated. The duration of the continuation-phase regimen hinges on the microbiological status at the end of the intensive phase of treatment; thus, obtaining a sputum specimen at the time of completion of 2 months of treatment is critical if sputum culture conversion to a negative result has not already been documented. The culture result of a sputum specimen obtained at the completion of the intensive phase of treatment (2 months) has been shown to correlate with

the likelihood of relapse after the completion of treatment for pulmonary TB albeit with low sensitivity. In patients treated for 6 months, having both cavitation on the initial chest radiograph and a positive culture at the completion of 2 months of therapy have been associated with rates of relapse of approximately 20%, compared with a rate of 2% among patients with neither factor.

In addition, the AFB stain can be used to confirm growth from various clinical specimens and can be used to release a patient from isolation after obtaining 3 negative AFB smears of sputum collected 8 h apart, one of which is an early-morning specimen (105). Although it is believed by some clinicians and investigators that there was a difference in staining of AFB when testing nonviable mycobacteria with carbol fuchsin-based stains versus fluorescence-based stains, Truant et al. (167) reported no major differences in staining characteristics with either heat-killed or antibiotic-killed AFB and viable AFB. Thus, an AFB smear alone cannot be used to determine the prestain viability of mycobacteria.

AFB Smear Preparation, Fixation, Staining, and Examination

Smears for AFB staining are best prepared from the sediment of specimens that have been liquefied, decontaminated, and concentrated by centrifugation. Inactivation of AFB smears that contain *M. tuberculosis* complex isolates for microscopy before the removal of the material from a BSC is an important safety factor in preventing the potential transmission of TB to technical staff. Heat fixing is one option, with the use of a slide warmer at 65°C to 75°C for at least 2 h once the slides are air dried (75). Alternatively, Chedore et al. (120) demonstrated that treatment with 5% phenol in ethanol for 5 min successfully fixed and inactivated all AFB smears containing *M. tuberculosis* complex isolates, both from concentrated sputum samples and from culture material. This chemical fixation protocol has the advantage of shortening turnaround times by 2 h for reporting AFB smear results.

Examination of two AFB smears will detect most AFB smear-positive cases. Testing of additional specimens provides only a marginal improvement in sensitivity (examination of a third sputum specimen increased the sensitivity between 2% and 5%) (164, 168). The efficiency of the concentration techniques used directly affects the sensitivity of the AFB smear. A systematic review of 83 studies demonstrated that sputum treated with bleach or NaOH and concentrated by centrifugation is, on average, more sensitive; however, the data were insufficient to conclude whether the gain in sensitivity would apply to persons living with HIV (168). Essentially, three major protocols are widely used for AFB smears: Ziehl-Neelsen and Kinyoun methods use carbol fuchsin as the primary stain, with subsequent examination by using light microscopy, while a third method uses a fluorescent dye, auramine O, alone or with rhodamine, with subsequent examination by fluorescence microscopy. Researchers from Johns Hopkins University evaluated a more rapid, commercially available auramine O stain that may be more cost-effective and efficient (169). A systematic review (164) showed that the sensitivity of fluorescence microscopy was 10% higher than that of conventional carbol fuchsin-stained sediments. In addition, it has been documented that the Kinyoun method of staining is less sensitive than Ziehl-Neelsen or fluorescent AFB staining (170). One hundred sixty-seven laboratories participated in a per-laboratory comparison of results. A comparison of prestained Ziehl-Neelsen versus prestained Kinyoun smears for the evaluation of differences in reading capabilities of participants revealed a significantly better performance of the Ziehl-Neelsen staining method ($P < 0.01$) (170). Similar results as to the lack of sensitivity of Kinyoun staining compared to routine, hot Ziehl-Neelsen staining under field conditions have also been reported (171).

Because acid-fast artifacts may be present in the AFB smear, morphology must be examined closely for the presence of long and typically slender acid-fast rods or coccobacilli that may appear slightly curved, beaded, isolated, in pairs, or in groups and stand out clearly against the background. Using auramine O stain, acid-fast organisms stain yellow-green (if auramine and rhodamine are used, AFB will fluoresce orange-yellow). Depending on the mycobacterial species, different morphologies may be

TABLE 11 Interpretation of AFB smears[a]

No. of AFB seen by carbol fuchsin stain at magnification of ×1,000	No. of AFB seen by fluorescent stain at magnification of[b]:		Interpretation according to:	
	×250	×450	CDC	WHO
0/smear	0/smear	0/smear	No AFB seen	No AFB seen
1–2/300 fields	1–2/30 fields	1–2/70 fields	Doubtful; repeat[c]	Confirmation required[d]
1–9/100 fields	1–9/10 fields	2–18/50 fields	1+	Scanty
1–9/10 fields	1–9/field	4–36/10 fields	2+	1+
1–9/field	10–90/field	4–36/field	3+	2+
>9/field	>90/field	>36/field	4+	3+

[a]See references 75 (CDC) and 177 (WHO). AFB, acid-fast bacilli.
[b]If using a different magnification, interpretations must be adjusted.
[c]Although counts of <3 AFB per 100 fields are not considered positive, a second AFB smear from the same sample may be stained and examined, or another specimen should be requested.
[d]Confirmation by another technician or by preparing, staining, and reading another AFB smear is required.

observed on stained AFB smears. For example, when stained from liquid medium, *M. tuberculosis* often demonstrates cording. Some NTM species can exhibit cording as well, albeit rarely, such as the MAC, *M. kansasii*, *M. gordonae*, *M. chelonae*, and *M. marinum* (172–174). NTM are often pleomorphic, sometimes appearing as long filaments or coccoid forms. An often challenging group of mycobacteria with respect to AFB stains is rapidly growing mycobacteria. These organisms may not always be acid fast with carbol fuchsin (1), auramine O (175), or auramine-rhodamine stain (175).

Of note, it is currently recommended that all positive AFB smears be confirmed either by a second reader, by restaining the slide (if a fluorescent stain was used) using a Ziehl-Neelsen stain, or by initially preparing two AFB smears, one for the fluorescent stain and the other for the Ziehl-Neelsen stain, in the event of a positive fluorescent AFB smear (132).

In general, with *M. tuberculosis* complex infections, the sensitivities of AFB smears vary due to a number of factors, such as the specimen type, the prevalence and severity of disease, the quality of specimen collection, the number of organisms in the specimen, and laboratory processing and staining procedures (170). Importantly, strict adherence to staining procedures, training and experience of laboratory personnel, and intermittent shedding of AFB can all impact the sensitivity of AFB smears. In general, if a patient is suspected of having pulmonary TB, a series of two or more sputum specimens with negative AFB smears makes this diagnosis very unlikely (115, 176, 177).

Interpretation and Reporting of Acid-Fast Smear Results

When acid-fast organisms are observed on an AFB smear, results must be quantified to be meaningful. Because this quantitation estimates the number of bacilli being excreted, the extent of a patient's infectiousness can be assessed for clinical and microbiological purposes. A positive AFB smear is reported by the laboratory, as are the staining method employed and the quantity of organisms. Recommended interpretations of and ways to report results are shown in Table 11.

Growth Detection

Solid mycobacterial cultures are generally held 6 to 8 weeks, and protocols for liquid media usually require a minimum of 6 weeks of incubation before cultures can be discarded as negative (1). Specimens with positive AFB smears that are culture negative should be held an additional 4 weeks. Culture-negative specimens that are positive for mycobacteria by one of the NAATs or for cases with a high suspicion of TB should also be held an additional 4 weeks (1).

In 2012, the CDC conducted a multicenter study to determine whether *M. tuberculosis* complex cultures in automated broth-based systems could reliably be considered negative sooner than 6 weeks (178). For laboratories using standard processing procedures, 100% of *M. tuberculosis* complex isolates were detected from initial and follow-up specimens in 28 and 35 days, respectively, and no yield of *M. tuberculosis*

complex bacteria on solid or MGIT liquid medium was observed after 5 weeks. Receipt of interim negative reports earlier than 6 weeks could assist clinicians in considering alternative diagnoses and could alter the timing and prioritization of public health interventions. According to the CDC study, laboratories using MGIT liquid medium could issue reports of no growth of *M. tuberculosis* complex bacteria on initial specimens as early as 4 weeks and, for patients undergoing treatment, as early as 5 weeks postinoculation. However, laboratories should analyze their own time-to-detection data to make informed protocol decisions.

Since many mycobacteria are slowly growing organisms, cultures can be examined for colony formation less frequently than routine bacterial cultures. All solid media should be examined within 3 to 5 days after inoculation to determine if there is contamination. In general, cultures should be examined twice per week for the first 2 weeks and then at weekly intervals. Liquid medium systems read cultures continuously and provide an alert when the culture turns positive for growth (1); however, MGIT tubes may be inspected manually, daily for up to 2 weeks and twice weekly or weekly thereafter (1).

Irrespective of the growth media, the acid fastness of the growth should be determined by AFB staining. Also, it is advisable to subculture the broth on a blood or chocolate agar plate to rule out contamination. Once growth is detected and confirmed as AFB, identification methods are performed (see Identification, below). AST can then be performed (1) (see Antimicrobial Susceptibility Testing, below). Positive cultures may be kept at temperatures of up to 35°C to 37°C for several weeks. The CLSI recommends that isolates needed for possible follow-up be frozen at −80°C (132).

IDENTIFICATION

Molecular Identification of Cultured Organisms

The molecular techniques used for the identification of mycobacterial organisms cultured from patient specimens include direct probe hybridization and sequence-based techniques. Probes are single-stranded oligonucleotides that can have various sizes but are generally less than 50 bp. Probe specificity is defined by the nucleic acid sequence of the probe. rRNA is a common target for identification by probes due to the relatively high copy number of rRNA in bacteria, thus increasing the sensitivity of detection. Furthermore, rRNA sequences contain conserved regions in addition to hypervariable regions, allowing the level of identification to be varied depending on the probe sequence. Commercially available probes for culture confirmation include those for the *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, the MAC, *M. gordonae*, and *M. kansasii* (AccuProbe; Hologic, San Diego, CA). Although probe-based detection and identification methods are more expensive than conventional culture and identification methods, they have increased accuracy and decreased turnaround times relative to conventional biochemical methods (179). However, both false-negative and false-positive results may occur. Bacterial strains may possess polymorphisms that prevent hybridization (180, 181), or there may be similar sequences among strains that result in cross-reactivity (182, 183). Additional disadvantages are the limited number of commercial probes available and the inability to probe clinical specimens directly. Although probes are not FDA cleared to detect and identify mycobacteria directly from MGIT broth, some laboratories have validated probes for this. Somoskovi et al. demonstrated 95.7% accuracy in identifying the *M. tuberculosis* complex when probes were used directly on positive MGIT broth cultures (184).

Line probe assays are available for certain NTM species, but they are not FDA cleared or FDA approved. Such tests include the INNO-LiPA Mycobacteria v2 assay (Fujirebio Europe, formerly Innogentics, Ghent, Belgium), GenoType CMdirect, GenoType NTM-DR, GenoType Mycobacterium CM, GenoType Mycobacterium AS (Hain Lifescience), Speed-oligo Mycobacteria (Vircell, Granada, Spain), and Nipro NTM+MDRTB detection kit 2.

In many larger laboratories, sequence-based identification has become a primary method to rapidly identify mycobacteria. This procedure can reduce the time to

identification over those of conventional methods, but initial growth of an isolate is still required prior to identification by sequencing. Sequencing may also be performed directly from a positive MGIT broth culture, but careful validation is required, and results should always be correlated with growth obtained from subculture. There are many targets used for sequence-based identification, but the 16S rRNA gene remains the most common target. The 16S rRNA gene encodes the highly conserved rRNA associated with the small subunit of the ribosome and is often used for taxonomic purposes and species identification. While 16S rRNA is highly conserved among bacteria, there are nucleotide variations concentrated in specific regions that are unique to each species. The entire gene is 1,550 bp, including the conserved and variable regions, but a discriminatory sequence can generally be obtained by using 500 bp (185). Universal primers complementary to the conserved regions permit the amplification of the gene from all bacterial species, and the resulting amplicon contains a unique sequence. There are commercially available kits for research-use-only sequencing of 16S rRNA (MicroSeq; ThermoFisher Scientific, Waltham, MA), but most clinical laboratories use laboratory-developed protocols. Notably, some mycobacteria are indistinguishable by 16S rRNA gene sequencing, including members of the *M. tuberculosis* complex, the *M. avium* subspecies, *M. chelonae* and *M. abscessus*, *M. kansasii* and *Mycobacterium gastri*, *M. marinum* and *M. ulcerans*, *M. senegalense* and *M. conceptionense*, *M. farcinogenes* and *M. houstonense*, *M. murale* and *M. tokaiense*, *M. mucogenicum* and *M. phocaicum*, *M. malmoense* and *M. szulgai*, *M. peregrinum* and *M. septicum*, and *M. porcinum* and *M. fortuitum*. Further differentiation may be required for some of these groups for epidemiological or clinical reasons. Most of these organisms can be differentiated by sequencing of other genes, such as the internal transcribed spacer (ITS), *rpoB*, *secA*, or *hsp65* (186–188). To separate the species within the *M. tuberculosis* complex, *gyrB* sequencing or deletion analysis is needed (113, 189, 190).

Sequence results are more robust than the results of conventional biochemical methods because they are less subjective given a comprehensive and accurate database for comparison. Analysis of the sequence data involves evaluating the quality of the sequence obtained and subsequent comparison of the sequence with known sequences through public and/or commercial databases such as NCBI GenBank, MicroSeq, the Integrated Database Network System (SmartGene, Raleigh, NC), or RipSeq (Isentio, Sunnyvale, CA). Since there are no FDA-cleared databases, users must carefully verify the performance of databases used in laboratory-developed protocols, as the quality of the databases varies. Based on CLSI recommendations, 100% identity is needed to identify an isolate to the species level. Identifications with 99.0 to 99.9% confidence should be reported as "*Mycobacterium*, most closely related to (species)." Isolates with 95.0 to 98.9% identity should be reported only to the genus level or with the comment "Unable to definitely identify by 16S rRNA gene sequencing, most closely related to *Mycobacterium* spp." (191). When possible, additional language should be included in the report regarding the *M. tuberculosis* complex. For example, if the *M. tuberculosis* complex can be ruled out, one should consider reporting the organism as "*Mycobacterium* spp. not *M. tuberculosis* complex."

MALDI-TOF MS

MALDI-TOF MS is being used with increasing frequency to identify mycobacterial isolates. MALDI-TOF MS detects the abundance of proteins with specific mass-to-charge ratios, which is displayed as a spectrum. The spectral data are then compared to a database to determine the likely identity of the organism. There are currently two commercially available MALDI-TOF MS systems in the United States, the MALDI Biotyper (Bruker Daltonics, Billerica, MA) and the Vitek MS system (bioMérieux, Durham, NC). The Vitek MS system has an FDA-cleared database, which includes mycobacteria (192). Laboratories using the MALDI Biotyper must build their own databases or rely on research-use-only databases for their laboratory-developed protocols (193). Like 16S rRNA gene sequencing, the accuracy of MALDI-TOF MS is dependent on both the robustness of the database and the quality of the spectra obtained. Obtaining quality

spectra can be difficult with mycobacteria due to their complex cell walls. Therefore, whole-cell preparations (i.e., applying a colony directly on a target plate) do not work for mycobacteria. A preextraction step must occur, usually involving bead beating or vortexing in ethanol, formic acid, and acetonitrile; the extracted proteins are then spotted onto the plate (194, 195). Importantly, the extraction procedure should be performed in a BSL-3 laboratory (or a BSL-2 laboratory using BSL-3 practices) and verified for its ability to effectively kill *M. tuberculosis* complex bacteria prior to moving the target plate to the mass spectrometer.

Several studies have demonstrated the ability of MALDI-TOF MS to identify clinically relevant rapidly growing mycobacteria, including *M. immunogenum*, *M. chelonae*, and *M. abscessus* (194–198). However, the subspecies of *M. abscessus* cannot be differentiated by MALDI-TOF MS. Slowly growing mycobacteria, including the MAC and the *M. tuberculosis* complex, have also been evaluated by MALDI-TOF MS. The *M. tuberculosis* complex, the members of which cannot be easily differentiated, can reliably be identified by MALDI-TOF MS, as can *M. avium*, *M. intracellulare*, *M. kansasii*, *M. xenopi*, *M. marinum*, and several other clinically relevant species (194–198). Of note, studies investigating slowly growing mycobacteria often use 14 to 21 days of growth to accumulate enough biomass for MALDI-TOF MS identification (194, 197). Unfortunately, this delay increases the time to reporting compared to what can be achieved with sequencing. Furthermore, the MALDI-TOF MS identification of mycobacteria has been studied mainly using isolates on solid media. Identification directly from broth media, such as BD MGIT or VersaTREK Myco broth, looks promising, but more studies are needed to determine the appropriate scores to use for the acceptability of species-level identifications (193, 196, 197). MALDI-TOF MS identification is relatively inexpensive to perform after the initial instrument expense (199). It takes about 90 min to obtain a MALDI-TOF MS result, compared to 1.5 days for sequence-based identifications.

HPLC

HPLC was first proposed for the identification of mycobacteria by the CDC in 1985 and was offered as a standard test at the CDC Mycobacteriology Reference Laboratory in 1990 (200, 201). HPLC can identify rapidly growing mycobacteria into groups or complexes but is not specific enough to identify most species. HPLC has been replaced in most laboratories by molecular methods (e.g., *rpoB* gene sequencing and MALDI-TOF) for more accurate species identification.

A commercial HPLC system, the Sherlock Mycobacteria Identification System (MYCO-LS) (MIDI Inc., Newark, DE), is an FDA-cleared HPLC method for the identification of mycobacteria. MYCO-LS identifies 25 species of mycobacteria by the analysis of mycolic acids. Like the CDC HPLC method, mycolic acids are extracted from unknown mycobacteria, and the HPLC mycolic acid profile is then compared to a library of reference strain profiles. The mycobacteria may be identified to the species or to the species-complex group level. Several studies have evaluated MYCO-LS and found that it is a rapid and accurate alternative method for mycobacterial species identification; however, it has a limited library of mycobacteria (202, 203).

Conventional Biochemicals

Identification of mycobacteria has traditionally relied on phenotypic characteristics such as the growth rate, colony morphology and pigmentation, optimal growth temperature, and reactions in a battery of biochemical tests. Many of these tests require growth on solid media, are poorly reproducible, and can take several weeks of incubation before species-level identification can be done (204). In addition, variability in phenotypic characteristics can be seen within strains of a species. With the development of molecular methods for the identification of mycobacteria, there are close to 200 recognized *Mycobacterium* species (6). Many of these new species have not been characterized biochemically and cannot be reliably identified by using biochemical methods.

It is now recommended that laboratories not rely on biochemical methods for the identification of *Mycobacterium* species (132, 205). Rapid molecular methods such as commercial DNA probes; sequencing of certain genes such as 16S rRNA, *rpoB*, *hsp65*, and other genes; as well as MALDI-TOF MS can now provide more accurate and rapid mycobacterial identification. If these capabilities are not available locally, isolates should be referred to a reference laboratory (205).

Lateral Flow Assays

Immunochromatography-based lateral flow assays using monoclonal antibody against the MPB64 protein (Rv1980c or RD2) secreted by the *M. tuberculosis* complex are reported to be reliable, rapid, and simple tools to identify the *M. tuberculosis* complex and differentiate its members from NTM in growth-positive solid or liquid cultures (206–209). At present, three commercial tests are available for routine clinical diagnostic use, the SD Bioline Ag MPT64 Rapid assay (Standard Diagnostics, Kyonggi-do, South Korea) (CE marked), the Capilia TB-Neo assay (Tauns, Numazu, Japan) (CE marked), and the MGIT TBc Identification test (Becton-Dickinson Instrument Systems, Sparks, MD, USA) (CE marked).

The Capilia TB-Neo assay is a second-generation test that eliminated previous cross-reactions with certain NTM and nonmycobacterial bacteria to improve specificity (210). Due to mutations (most commonly due to a 63-bp deletion at position 196) in the gene encoding MPB64, the test gave false-negative results for some *M. bovis* BCG and, more recently, *M. africanum* variants (211–213). According to a recent meta-analysis, the overall sensitivity, specificity, and positive and negative predictive values of the Capilia TB assay (first generation) for clinical specimens were 98.8%, 99.1%, 99.4%, and 98.2%, respectively; those of SD Bioline were 97.0%, 100%, 100%, and 87.2%, respectively; and those of MGIT TBc were 97.9%, 99.5%, 99.7%, and 96.8%, respectively (214).

Completion of the assay requires a BSL-3 laboratory and manipulation in a BSC. However, SD Bioline was noninferior regarding performance characteristics when performed on heat-inactivated (1 h at 80°C) growth-positive MGIT broth, which offers the possibility of performing the test on these inactivated samples outside a BSC (Loganathan Prabakaran, FIND India Office, personal communication).

ANTIMICROBIAL SUSCEPTIBILITY TESTING

The determination of antimicrobial resistance in mycobacteria is important for patient treatment, and with the *M. tuberculosis* complex, it is essential not just for individual patient treatment but also for TB control. AST information guides treatment, which is especially vital with the emergence of drug-resistant strains and for estimating the prevalence of drug resistance in a community. Phenotypic, culture-based techniques for AST rely on the isolation of the organism, and although there are some molecular methods available, phenotypic testing must still be performed. Nevertheless, there are a limited number of methods available and even fewer that are FDA cleared for the U.S. market.

This section discusses the available AST methods for *M. tuberculosis* complex and NTM. If AST for the *M. tuberculosis* complex cannot be performed in-house, the specimen and/or isolate should be referred to a laboratory that has the capability to perform this testing, such as a local or state public health laboratory. The CDC Division of TB Elimination Laboratory Branch and some reference laboratories also provide certain reference AST services.

Antimicrobial Susceptibility Testing for the *Mycobacterium tuberculosis* Complex

AST for the *M. tuberculosis* complex should be performed on at least the initial isolate from each patient with TB. AST can be repeated after 3 months or earlier, if a patient remains culture positive and/or is not responding to treatment (98, 115). If the *M. tuberculosis* complex is isolated from a different specimen source, it may also be appropriate to perform AST on an isolate from that different source. Once a culture has been confirmed as *M. tuberculosis* complex, AST should be performed by a growth-

TABLE 12 Critical concentrations for testing *M. tuberculosis* in solid and liquid media[a]

Drug	Critical concn of drug (μg/ml)				
	LJ medium[b]	7H10 agar[b]	7H11 agar[b]	MGIT960[b]	VersaTREK[c]
Isoniazid[d]	0.2	0.2 (1.0)	0.2 (1.0)	0.1 (0.4)	0.1 (0.4)
Rifampin	40.0	1.0	1.0	1.0	1.0
Ethambutol	2.0	5.0	7.5	5.0	5.0
Pyrazinamide				100.0	300.0
Streptomycin	4.0	2.0	2.0	1.0	
Amikacin	30.0	4.0		1.0	
Kanamycin	30.0	5.0	6.0	2.5	
Capreomycin	40.0	4.0		2.5	
Ethionamide	40.0	5.0	10.0	5.0	
Cycloserine	30.0				
PAS	1.0	2.0	8.0	4.0	
Ofloxacin	4.0	2.0	2.0	2.0	
Levofloxacin		1.0		1.5	
Moxifloxacin[e]		0.5/2.0		0.5/2.0	
Linezolid				1.0	

[a]LJ, Löwenstein-Jensen; 7H10 agar, Middlebrook 7H10 agar; 7H11 agar, Middlebrook 7H11 agar; PAS, *para*-aminosalicylic acid.
[b]See reference 221.
[c]See reference 343.
[d]Some laboratories may test an additional higher isoniazid concentration (shown in parentheses) to differentiate between low- and high-level isoniazid resistance.
[e]Two concentrations are proposed by the WHO. In programs using ofloxacin or levofloxacin and moxifloxacin, possible testing is for moxifloxacin only at both concentrations (0.5 and 2.0 μg/ml) or test ofloxacin or levofloxacin at its appropriate level, and moxifloxacin at the higher concentration (2.0 μg/ml). In programs using ofloxacin or levofloxacin only, test only these drugs. For programs using only moxifloxacin, testing is performed with the higher concentration (2.0 μg/ml) of moxifloxacin only.

based method. This method should include, at a minimum, AST for the first-line antituberculous drugs RIF, INH (2 concentrations), EMB, and PZA. If RIF resistance or resistance to any two of these drugs is detected, testing for susceptibility to additional, second-line drugs should be performed.

Phenotypic methods for AST. Most growth-based conventional methods test the susceptibility of the microorganism to a critical concentration of the drug. The critical concentration (Table 12) represents the lowest concentration of the drug that inhibits 95% of wild-type strains of *M. tuberculosis* that have never been exposed to anti-TB drugs and does not inhibit patient strains that are considered to be resistant. Essentially, the critical concentration differentiates antimicrobial-susceptible from antimicrobial-resistant strains and is based on international convention (215, 216). For some drugs, e.g., RIF, the critical concentration is well established, but for other drugs, there are fewer data to support a critical concentration, and for certain drugs, it can be difficult to determine low-level resistance. Another AST method, broth microdilution, can be used to determine a MIC for each drug. The MIC represents the lowest concentration of an antimicrobial, in a series of dilutions of a drug, that will inhibit the visible growth of a microorganism (217). Cambau et al. (218) utilized MGIT960 instrumentation equipped with TB eXiST software to establish quantitative AST for *M. tuberculosis* and compared the results with those of genotypes associated with drug resistance.

AST by growth-based methods should always be performed on a pure *M. tuberculosis* complex culture, as contaminating organisms can affect susceptibility results. When AST is performed, the bacteria should be inoculated onto solid media such as Middlebrook 7H10 or 7H11 agar as well as blood agar plates to determine colony morphology and to check for purity. In cases where cultures are mixed, agar plates are useful for identifying and separating *M. tuberculosis* complex and NTM because organisms can be picked and reisolated for purity.

The indirect AP method is considered the reference standard method for phenotypic AST of *M. tuberculosis* complex isolates. However, it is a labor-intensive method, with the calculation of resistance by performing colony counts, which requires skilled staff.

In addition, there is a longer turnaround time, up to 3 weeks, than those of broth-based methods. The indirect AP method is not commonly used for first-line drugs in diagnostic laboratories for these reasons, and commercial broth-based methods are favored in the clinical laboratory setting and recommended for use by the CLSI. The following section details common commercial broth-based methods. For those methods that use critical concentrations, they are based on comparison to indirect AP and are defined as equivalent critical concentrations (219). To provide susceptibility data to health care providers earlier, some laboratories perform a direct AP method on the processed AFB-positive sediment once the AFB are identified as belonging to the *M. tuberculosis* complex.

The Bactec MGIT 960 method (Becton Dickinson, Sparks, MD) is used for the growth and detection of mycobacteria as well as AST. For AST, MGIT tubes containing the critical concentrations of drugs (Table 12) can be inoculated from solid or liquid *M. tuberculosis* complex isolates. For inoculation procedures, the manufacturer's instructions must be followed. A categorical susceptible or resistant result is provided. Some laboratories may use EpiCenter software in order to monitor growth dynamics (220). This method is FDA cleared for RIF, INH, EMB, PZA, and streptomycin. Other drugs may be tested as a laboratory-developed test (221). If there are any other deviations from the manufacturer's instructions, the test must be verified in-house as a laboratory-developed test.

The VersaTREK method is an automated system used for the growth and detection of mycobacteria as well as AST. Tubes containing critical concentrations of the drugs (Table 12) can be inoculated with solid or liquid cultures, according to the manufacturer's instructions. This method is FDA cleared for RIF, INH, EMB, and PZA, and other drugs may be tested as laboratory-developed tests. A categorical susceptible or resistant result is provided.

The TREK Sensititre method (ThermoFisher Scientific, Oakwood Hills, OH) is a semiautomated research-use-only broth microdilution method for susceptibility testing of mycobacteria in a microtiter plate format. This method uses a 96-well plate that is inoculated with a series of dilutions of first- and second-line drugs (a total of 12 drugs are provided, each with a minimum of 7 dilutions on the standard plate). Microtiter plates can be inoculated from solid or liquid cultures. The plate is incubated and read at intervals for visible growth and determination of the MIC, according to the manufacturer's instructions, at 7, 10, 14, and 21 days. The standard plate tests for resistance to the following drugs: RIF, INH, EMB, streptomycin, rifabutin, ethionamide, amikacin, kanamycin, ofloxacin, moxifloxacin, cycloserine, and *para*-aminosalicylic acid. This test determines MIC values, but there are no guidelines for the interpretation of susceptibility or resistance results; however, this information, when considered in comparison with the established critical concentration of a drug and its achievable serum concentration in healthy individuals, can be a valuable tool to assess the degree of resistance and, in turn, guide the adjustment of treatment.

If AST for the *M. tuberculosis* complex is not available, specimens or isolates should be sent to a reference laboratory for testing. The capabilities of public health laboratories vary from state to state, but many states have laboratories that are able to provide AST for the *M. tuberculosis* complex or are able to refer specimens to the public health AST reference laboratory (California Department of Public Health Laboratory) or to the CDC (222). If testing is performed in-house, it is important to have a robust quality assurance program, which includes elements such as quality control, proficiency testing and performance monitoring (as discussed in Quality Assurance, below), and appropriate biosafety practices (as discussed in Biosafety, above). For AST, quality control should include the use of a fully susceptible isolate (e.g., *M. tuberculosis* strain H37Rv). With caution, a drug-resistant strain may be included; however, a resistant strain should not be resistant to more than two drugs, as this poses safety concerns according to the CLSI (219). Quality control organisms should be included whenever there is a new lot or shipment, weekly, or every run (depending on the manufacturer's instructions or according to a laboratory's IQCP). Drug resistance rates should be

TABLE 13 Commonly used phenotypic methods for AST of the *M. tuberculosis* complex in the United States

Growth-based method	Format	Comments
Indirect agar proportion method	Critical concn; solid media (Middlebrook 7H10 or 7H11 agar); turnaround time of ~1 mo from setup date	Criterion "gold standard"; complex method; pyrazinamide testing not available; long turnaround time; laboratory-developed test; not commercially available
Bactec MGIT320 or MGIT960 mycobacterial detection system (Becton-Dickinson)	Critical concn; liquid media (MGIT); turnaround time of 4–13 days from setup date	Reported issues with ethambutol critical concn and false pyrazinamide resistance[a]; FDA cleared for first-line drugs
VersaTREK (ThermoFisher Scientific)	Critical concn; growth matrix liquid/sponge; turnaround time of 4–13 days from setup date	FDA cleared for first-line drugs
Sensititre (ThermoFisher Scientific)	MIC; liquid media; turnaround time of 14–21 days from setup date	PZA testing not available; no MIC interpretive criteria; research-use-only test

[a]See references 344 and 345.

monitored to detect unusual increases or decreases in the rates, which may indicate problems with AST. Proficiency testing should be performed for quality assurance and is available for first-line drugs through the CAP, the American Proficiency Institute (API), and Accutest (summarized in Table 3). The CDC also provides a voluntary Model Performance Evaluation Program for the evaluation of test performance that includes strains with various resistance patterns. (https://www.cdc.gov/tb/topic/laboratory/mpep/).

A summary of the most commonly used growth-based methods in the United States is provided in Table 13.

Molecular AST methods. Molecular methods detect mutations in genes associated with resistance to anti-TB drugs. These methods are rapid and specific, but phenotypic AST must still be performed, as genotypic resistance may not always translate to phenotypic resistance. If they are available, molecular AST methods should be used in an AST algorithm since they can provide timely information to the clinician about drug resistance and may be superior to broth-based AST, e.g., for PZA (223, 224). If molecular methods for the detection of resistance are not performed, the specimen/isolate may be referred to a laboratory that has the capability to perform this testing, such as a reference laboratory or a local or state public health laboratory. The CDC provides certain AST services, including the MDDR program (http://www.cdc.gov/tb/topic/laboratory/guide.htm), which provides molecular testing for resistance to a range of first- and second-line drugs (112).

The Xpert assay is an FDA-cleared, rapid, real-time PCR test that simultaneously detects the presence of the *M. tuberculosis* complex and mutations in the RRDR of *rpoB*. RIF resistance detected by Xpert requires confirmation by additional AST methods (156) (see "Detection of Antimicrobial Resistance," above, for more information on the molecular detection of drug resistance and Table 10 for interpretation and reporting of Xpert results). Since many clinical laboratories may have an Xpert instrument for other tests, the implementation of the moderately complex Xpert test makes screening for RIF resistance more widely available. Note that testing of specimen types other than sputum must be carefully verified as a laboratory-developed test for off-label usage (225).

The GenoType MTBDR*plus* Version 2 assay is a PCR-based line probe assay for the detection of the *M. tuberculosis* complex, mutations in the *rpoB* gene associated with resistance to RIF, and mutations in the *inhA* and *katG* genes associated with resistance to INH. This rapid test can be performed directly on the specimen or from culture isolates (184, 226). An additional assay, MTBDR*sl* Version 2 (Hain Lifescience, Nehren, Germany), is also available, which detects mutations in the *gyrA*, *gyrB*, *rrs*, and *eis* genes associated with resistance to FQ and aminoglycosides-cyclic peptides (227). Nipro Corporation (Japan) has developed a line probe assay (Nipro NTM+MDRTB) that is like that of Hain Lifescience. This assay allows the detection of RIF and INH resistance-conferring mutations, the identification of the *M. tuberculosis* complex, and the identification of some common NTM, including *M. avium*, *M. intracellulare*, and *M. kansasii*

TABLE 14 Summary of some molecular methods for *M. tuberculosis* complex AST

Molecular method	Format	Description
Xpert MTB/RIF (Cepheid)	Real-time PCR; detects mutations in the *rpoB* gene; turnaround time of 2 h	FDA cleared; rifampin resistance only; ease of use
GenoType MTBDR*plus* and MTBDR*sl* (Hain Lifescience)	Line probe assay; MTBDR*plus* detects mutations in the *rpoB*, *inhA*, and *katG* genes; MTBDR*sl* detects mutations in the *gyrA*, *gyrB*, *rrs*, and *eis* genes; turnaround time of 6 h	Research use only (U.S.); rifampin and isoniazid on the same line probe assay; additional assay for fluoroquinolones and aminoglycosides (amikacin or kanamycin)-cyclic peptides (capreomycin); interpretation of test results and result reporting require laboratory expertise; type of mutation is important and may direct additional testing necessary for patient treatment
Sequencing (Sanger sequencing and pyrosequencing)	DNA sequencing; detects mutations in target genes; turnaround time of $<$24 h	Laboratory-developed test; able to detect mutations in any targeted genes; pyrosequencing for shorter sequences only (e.g., not *pncA*); interpretation of test results and result reporting require laboratory expertise; type of mutation is important and may direct additional testing necessary for patient treatment

(228). These tests are research-use-only tests in the United States but are CE marked for use in Europe. Other CE-marked assays include the Autoimmun Diagnostika GmbH TB Isoniazid, Rifampin; TB Aminoglycoside; and TB Fluoroquinolone, Ethambutol kits (229).

Sequencing methods can be used to target a variety of genes to determine mutations associated with resistance. Sanger sequencing can be used for any DNA sequencing, e.g., the *pncA* gene to detect mutations associated with PZA resistance. Since pyrosequencing methods are used for shorter DNA fragments, this method is not useful for the *pncA* gene, where sequencing of the 670-bp amplicon is required. These sequencing methods are laboratory-developed tests but can still play an important role in AST for the *M. tuberculosis* complex (230, 231). An example of when such sequencing may be necessary is when performing the Sensititre MIC method for phenotypic AST. This method does not allow testing with PZA since it does not have the acidic conditions required for the growth-based testing of this drug. Sequencing and identifying mutations in the *pncA* gene can provide information on the potential for PZA resistance. However, not all PZA resistance is determined by the *pncA* gene and not all mutations in the *pncA* gene are relevant, and additional phenotypic testing may still be needed (223). Next-generation sequencing technologies are advancing at a rapid pace and are beginning to play roles in identification, AST, and more (223, 232, 233).

Quality assurance considerations for molecular methods include proficiency testing and performance evaluations (234), especially the potential for false-positive results for tuberculosis in low-prevalence populations like that of the United States; similarly, false RIF resistance has been reported (235, 236). The only CMS-approved proficiency testing program available exclusively for *M. tuberculosis* PCR and molecular detection of RIF resistance is available through the Wisconsin State Laboratory of Hygiene proficiency testing service. A summary of some of the molecular methods is provided in Table 14.

Antimicrobial Susceptibility Testing for Nontuberculous Mycobacteria

The presently accepted recommendations and guidelines for *in vitro* AST of clinically significant NTM isolates are summarized in recommendations of the ATS and IDSA from 2007 (52) and in guidelines of the CLSI from 2011 (219). A review by Brown-Elliott and colleagues (54) provides additional important recommendations and updates.

The role and relevance of *in vitro* AST of NTM to guide the treatment and clinical management of patients with NTM disease are under continuous debate. The basis of this debate is that, in contrast to the *M. tuberculosis* complex, the clinical response to antimycobacterial drugs or antibiotics has been shown to correlate with only some compounds and in only some NTM (e.g., the MAC and macrolides and amikacin, *M.*

kansasii and RIF, *M. marinum*, and *M. fortuitum*), whereas similar correlations for several other clinically significant NTM (e.g., *M. abscessus*) were not or could not be established. On the other hand, most clinically significant NTM isolates already show intrinsic resistance or high *in vitro* breakpoints for several antibiotics upon first isolation (54). Therefore, AST on NTM seems to be logical and necessary to accumulate data on *in vitro* AST patterns for NTM. However, it is important to keep in mind that performing AST on clinically nonsignificant NTM isolates is a waste of time and resources, and results may be misleading for patient management. Because most NTM are ubiquitous in soil, water, and other environmental sources, determination of the clinical significance of an isolate is warranted before initiating any AST. Additionally, AST results for NTM can be method and species dependent.

Rapidly growing NTM. The gold-standard method for AST of rapidly growing NTM is a broth microdilution assay; a commercial research-use-only product is the TREK Sensititre MIC plate. At this time, there are no FDA-cleared platforms for NTM AST. AST of rapidly growing NTM should have rigorous quality control using CLSI-recommended reference strains to ensure not only quality testing but also reproducibility of MICs within the recommended and acceptable ranges for antimicrobials tested. MIC results for imipenem, meropenem, and tetracycline may be invalid after more than 5 days of incubation because of stability-related problems with these drugs (54). Isolates that are susceptible to clarithromycin should be further incubated for 14 days to rule out inducible macrolide resistance, which is a common phenomenon due to the presence of an rRNA methylase *erm* gene present in most clinically significant rapidly growing NTM.

To decrease the turnaround time and save resources on prolonged incubation, routine DNA sequencing of particular *erm* genes, such as *erm*(41) in *M. abscessus*, may facilitate both the rapid detection of macrolide-inducible resistance and subspecies identification of *M. abscessus* isolates (82, 237). Hain GenoType NTM-DR Ver 1.0 can detect macrolide resistance [*erm*(41)] and subspecies of *M. abscessus* within 5 h and is CE marked in Europe (238). Breakpoints have not been established by the CLSI for additional methods, such as agar disk diffusion and Etest (bioMérieux, Durham, NC), or have shown problems with reproducibility (54), and therefore, these methods are not recommended for AST of rapidly growing NTM.

Slowly growing NTM. For slowly growing NTM, the CLSI-recommended method for AST is a broth-based assay, except for *M. haemophilum*, for which the recommended method is an agar disk elution that requires more prolonged incubation (54, 219). Hain GenoType NTM-DR Ver 1.0 can detect resistance to macrolides and aminoglycosides and identify MAC isolates to the species level and is CE marked in Europe. Recommendations of the ATS/IDSA (52) and the CLSI (219) for AST of slowly growing NTM include the following. Clarithromycin susceptibility testing is recommended for new and previously untreated MAC isolates. No other drugs are recommended for AST of new and previously untreated MAC isolates. There is no recognized value for testing of MAC isolates against first-line antituberculosis agents. Clarithromycin susceptibility testing should be performed for MAC isolates from patients who fail macrolide therapy or prophylaxis. Previously untreated *M. kansasii* isolates should be tested against rifampin only. Isolates of *M. kansasii* that show susceptibility to rifampin will also be susceptible to rifabutin. *M. kansasii* isolates that are resistant to rifampin should be tested against a panel of secondary agents, including rifabutin, ethambutol, isoniazid, clarithromycin, fluoroquinolone, amikacin, sulfonamides, and linezolid. *M. marinum* isolates do not require susceptibility testing unless the patient fails treatment after several months. There are no current recommendations for one specific method of *in vitro* AST for fastidious NTM species (e.g., *M. haemophilum*) and some less commonly isolated NTM species. Verification and quality control must be in place for AST of all species of NTM.

Considerations about Antimicrobial Susceptibility Testing for Mycobacteria

Many laboratories do not have the capability or have a limited capability to perform AST and, as discussed in this section, may need to refer specimens and isolates to other

laboratories. There are a few considerations when referring specimens and/or isolates. First, submitting and referral laboratories should be familiar with shipping guidelines for infectious substances. Second, laboratories should consider the techniques that are used at the referral laboratory for AST, the drugs that are tested, the turnaround time, and reporting mechanisms. Third, laboratories should consider referring specimens for molecular *M. tuberculosis* complex AST, especially if resistance is suspected. Even if a laboratory has culture capabilities, specialized laboratories and the CDC can perform molecular AST on the specimen and provide susceptibility data to health care providers earlier than with growth-based AST. Laboratories should also consider referring isolates for *M. tuberculosis* complex AST if they do not have the capability in-house. Ideally, mycobacteria that have been identified can be referred by using primary liquid cultures (rather than waiting for solid medium, although this can also be sent). Finally, AST for slowly and rapidly growing NTM is offered in only a few reference or public health laboratories, and these laboratories may test additional compounds on a research-use-only basis.

A common challenge with the *M. tuberculosis* complex is discordant results, especially when testing is performed by multiple, complex techniques and at more than one laboratory. There are several reasons for discordant results, including but not limited to the following: the culture is not pure; the isolate itself may consist of subpopulations; comparison of results from different methods, e.g., phenotypic versus genotypic, or testing performed on different media (with different inocula and/or different critical concentrations), is difficult; methods are not standardized; the result is an MIC, and the result may be close to the critical concentration; and laboratory error with mislabeling or contamination is possible (115). Van Deun et al. (239) described discordant antimicrobial susceptibility results between growth-based and molecular testing during a proficiency testing event. In that study, it was concluded that low-level but probably clinically relevant RIF resistance linked to specific *rpoB* mutations can be missed by growth-based methods. A guide was reported by Hofmann-Thiel et al. (240) providing suggestions for investigating discordant molecular test results, including results of Xpert and line probe assays and growth-based assays for rifampin resistance.

QUALITY ASSURANCE

High-quality clinical laboratory testing is an integral part of providing quality health care. Quality assurance programs and indicators ensure that laboratories monitor and improve their clinical testing services through ongoing analysis and assessment. This section discusses several quality assurance features that laboratories should consider integrating into their quality management program in the mycobacteriology laboratory. Within the quality plan, several parameters should be addressed, including general laboratory systems and preanalytical, analytical, and postanalytical phases of testing.

CLSI document QMS12-A provides laboratories general suggestions on developing key quality indicators in the laboratory setting (241). A clear process that addresses the development and use of quality indicators is effectively described in this document, along with other meaningful tools related to process improvement.

Specimen Collection

A robust quality assurance program begins with adequate specimen collection. It is incumbent on the laboratory to appropriately communicate specimen requirements to the health care provider to ensure the best-quality outcome for the patient. This communication should be regular in some capacity, since providers change and knowledge can be forgotten. Provider instructions are a crucial component to ensure that adequate specimens are collected, labeled, and transported appropriately for processing. Communication with the provider as to which specimens will be rejected is important and should be reinforced on an ongoing basis. The optimal specimen collection volume for sputum is 5 to 10 ml (75). It is known that the volume of sputum can directly impact the ability of the laboratory to recover and isolate mycobacteria from a specimen and that submission of a specimen of $<$3 ml may negatively impact

organism recovery. If specimens that are <5 ml are routinely submitted, there should be a mechanism in the laboratory to monitor appropriate specimen collection (see "Acceptable Specimens and Rejection Criteria," above), and the laboratory should consider a quality monitor to assess specimen volume and have a mechanism for provider feedback if specimen requirements are not being followed (103). It is recommended that the provider be notified of unsatisfactory specimens as soon as possible or within 24 h. Instructions to the provider should clearly state that sputum from deep in the respiratory tract is the optimal specimen, as opposed to saliva. Monitoring the number of specimens per patient can also be part of a robust quality assurance program. In larger systems, tracking the numbers of specimens per patient could be onerous unless an electronic mechanism to do so is readily available.

Critical to patient care and management from an infection control perspective is the laboratory's role in the timely processing and reading of AFB smears. The laboratory must process specimens as soon as possible after collection and/or arrival in the laboratory and provide AFB smear results within 24 h of receipt of the specimen in the laboratory (103, 242). Specimen delivery can also be broken down into many segments to monitor how effectively specimens are being transported to the laboratory for processing, especially if the laboratory is in a remote location compared to either the hospital or outpatient facilities. The faster a result is provided to the health care provider, the sooner the patient can be appropriately treated, which will augment patient care, prevent the unnecessary spread of infection to others, and/or allow the patient to be released from isolation sooner (103, 158).

Another quality assurance indicator in the mycobacteriology laboratory is providing appropriate feedback to the health care provider. This not only will enhance quality and patient care but also can positively impact the laboratory so that time is not wasted processing inappropriately submitted specimens (243). Since many health care institutions have electronic medical records, direct feedback about specimen rejection via the hospital information system can be a rapid way to educate the provider.

Specimen Processing and Decontamination

Specimen management is very important across all clinical laboratory disciplines. Within the mycobacteriology laboratory, it is generally accepted that to maintain adequate proficiency for culturing specimens for AFB, a minimum of 20 specimens should be processed and cultured per week (103). If these numbers cannot be met, laboratories should consider referring specimens to a reference laboratory for testing.

For those laboratories that process and culture AFB specimens, cross-contamination with the *M. tuberculosis* complex is one event that can have serious consequences for the patient. After an extensive review performed in 2000, it was determined that the median false-positive rate due to cross-contamination in the mycobacteriology laboratory was approximately 3.1% (244). A cross-contamination event not only can result in a patient being treated with an inappropriate course of antibiotics but also can mask the true underlying condition or disease of the patient (245, 246). Laboratory practices should be in place not only to mitigate cross-contamination but also to uncover a contamination event if it occurs. The integrity of the laboratory rests on mitigating these events. The laboratory understanding of a root-cause analysis can be crucial to investigations related to contamination events (132).

There are several steps/processes that can be integrated into the specimen-processing procedure that will reduce errors associated with cross-contamination. These steps/processes can include, but are not limited to, the following: (i) supplementing broth cultures with the appropriate antibiotic cocktail prior to inoculating the sample aliquot; (ii) performing all work on towels soaked with an appropriate disinfectant to minimize droplets from the specimen contaminating surfaces or other materials; (iii) opening and processing one patient sample at a time within the BSC; (iv) using "single-use" reagents such as buffers that are part of the decontamination procedure; (v) if using Pasteur or serological pipettes, using only pipettes that have cotton plugs, which can reduce contamination of equipment associated with pipetting steps; (vi)

performing follow-up on specimens from first-time-positive AFB patients that are processed in the same batch as specimens of other known positive patients; and (vii) as recommended by the WHO Global Laboratory Initiative, including one negative-control specimen and one positive-control specimen weekly with a batch of clinical specimens (134). However, the inclusion of a positive control in the processing of patient specimens is discouraged due to the risk of cross-contamination (1). To resolve whether a cross-contamination event has occurred, laboratories can send out the isolates from the single-positive patient and the sentinel (possible source specimen from a known positive patient) for genotyping. Laboratories can refer these isolates to their state or local public health laboratory, which will forward the isolates to a CDC-contracted laboratory for genotyping. This definitive approach can truly make a difference in patient management and improve the quality of testing in the laboratory. An extensive list of measures related to specimen processing and decontamination can be found in the 2013 Association for Public Health Laboratories document *Mycobacterium tuberculosis*: *Assessing Your Laboratory* (103).

Culture Growth

There are several indicators to assess the efficiency of the decontamination and digestion procedures, since these steps ultimately affect organism recovery. Contamination of media for culture growth should be monitored within the mycobacteriology laboratory. Some contamination of culture medium is expected, but a general approximation is that contamination rates are 2 to 5% for solid media and 7 to 8% for liquid media containing antimicrobials (140, 247). There is a balance between mild decontamination, which could result in the overgrowth of non-AFB bacteria and interfere with the isolation of the true pathogen, and, conversely, harsh decontamination, which can lead to false-negative AFB cultures. Monitoring the contamination rate assists in ensuring that digestion and decontamination procedures are adjusted appropriately (75). If a contamination rate of >5 to 8% is observed, monitoring the time of transport of the specimen to the laboratory may be prudent, as delays in specimen submission may be occurring. The longer it takes to transport the specimen to the laboratory, the more time the normal flora has to multiply and overgrow AFB. Of note, if only a few colonies of non-AFB bacteria are contaminating an agar plate or slant, this should not be counted as contamination or overgrowth if the ability to isolate the pathogen is not impeded. Solid medium that is completely overgrown or liquefied, thereby making the isolation of AFB impossible or severely compromised, is considered contaminated. It is also important to monitor specimens submitted from CF patients, which warrant a different decontamination procedure (75). See the section Work-Up of Specimens for these procedures.

Tracking results for a defined patient population can be a very useful quality monitor (248), since an increase in the AFB positivity rate can alert the laboratory of a potential outbreak or pseudo-outbreak within the health care institution. However, if the positivity rate decreases compared to anticipated percentages, this might mean that the decontamination procedure is too stringent, resulting in false-negative cultures. All these quality monitors are important for assessing the robustness of the processes and procedures in the mycobacteriology laboratory (103).

For those laboratories performing culture, it is critical that correlation analysis of AFB smear and culture results be performed. Excessive numbers of AFB smear-positive/culture-negative specimens warrant a quality assurance investigation. Findings may include contamination with AFB in the test system, such as contaminated bronchoscopes (51) or contaminated water in general. Mycobacteria can be present in water, and autoclaving does not necessarily destroy the acid-fast properties of an organism. Too harsh of a decontamination procedure could also be the cause of a higher rate of AFB smear-positive, culture-negative specimens (132). The competency of the technologist reading the AFB smears should also be considered.

Finally, numbers of NAAT-positive, culture-negative specimens should be monitored to determine if the decontamination procedure may be too stringent and is killing the

TABLE 15 Suggested quality assurance monitors in the mycobacteriology laboratory[a]

Area	Quality assurance monitor[b]
Specimen collection	Monitor vol of specimens submitted; provide feedback to providers
	Monitor no. of specimens per patient; electronic mechanism for larger laboratories would be necessary
	Monitor rejected specimens
Turnaround time	Monitor specimen delivery turnaround time
	Monitor turnaround time for processing specimen
	Monitor turnaround time for reporting AFB smear results
	Monitor turnaround time for reporting first-line AST results for the *M. tuberculosis* complex
Cross-contamination	Monitor first-time-positive TB patients; send isolates for molecular typing if contamination is suspected
Processing of specimen	Monitor robustness of digestion/decontamination procedure by monitoring non-AFB growth in solid and liquid media
	Monitor positivity rate in population
	Correlate AFB smear positivity with culture results
	Correlate TB NAAT positivity with AFB smear and culture results

[a]See reference 248.
[b]AFB, acid-fast bacilli; TB, tuberculosis; NAAT, nucleic acid amplification test.

mycobacteria in the specimen or if the NAAT result is false positive due to contamination. Wipe testing (sentinel testing) for contamination should be performed periodically (249), or at the director's discretion, in all laboratories performing molecular testing to document that the environment is clean. NAAT-negative, culture-positive specimens could indicate a contamination event in the processing/culture area of the laboratory, so it is important to investigate this as well.

In conclusion, there are several approaches to assessing and monitoring quality within the mycobacteriology laboratory. Consultation with other colleagues is also prudent when looking for new avenues for continuous quality improvement, in addition to many of the references in this section. Table 15 lists several quality monitors that may be appropriate for laboratories that test specimens for mycobacteria, and benchmarks can be found in Table 4.

USE OF INTERFERON GAMMA RELEASE ASSAYS FOR DIAGNOSIS OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION

In 2010, the CDC reported updated guidelines (250) for the use of IGRAs as aids in diagnosing both LTBI and active TB disease, and in 2014, the American Academy of Pediatrics released a technical report entitled *Interferon-Gamma Release Assays for Diagnosis of Tuberculosis Infection and Disease in Children* (251). The primary purpose of IGRAs is to identify those who will benefit from LTBI therapy. Prior to 2001, the TST was the only immunological test for TB infection approved in the United States. In 2001, the Quantifier-TB (QFT) test (Cellestis Limited, Carnegie, Victoria, Australia) was the first IGRA approved by the FDA (250). In 2005, the QuantiFERON-TB Gold (QFT-G) test was FDA approved (252). Previous guidelines were reported in 2003 for the use of QFT (253) and in 2005 for the use of QFT-G (252). Since 2005, two new IGRAs have been FDA approved: the QuantiFERON-TB Gold In-Tube (QFT-GIT) test (Qiagen, Germantown, MD [formerly Cellestis Limited]) and the T-Spot.TB (T-Spot) test (Oxford Immunotec Limited, Abingdon, United Kingdom) (250). These tests may improve specificity and differ from previous IGRAs in the antigens used, methods, and interpretive criteria. CDC guidelines reported in 2010 address the use of these newer IGRAs (250).

The ATS/IDSA/CDC clinical practice guidelines reported in 2017 (98) recommend performing an IGRA instead of a TST for individuals 5 years of age or older who meet the following criteria: the individuals are likely to be infected with the *M. tuberculosis* complex, the individuals have a low or intermediate risk of disease progression, it has been decided that testing for LTBI is warranted, and the individuals either have a history of BCG vaccination or are unlikely to return to have their TST results read. Additionally, the guidelines suggest performing an IGRA rather than a TST for all other individuals 5 years of age or older who are likely to be infected with the *M. tuberculosis* complex, who have a low or intermediate risk of disease progression, and for whom it has been decided that testing for LTBI is warranted. There are insufficient data to

recommend a preference for either a TST or an IGRA as the first-line diagnostic test for individuals 5 years of age or older who are likely to be infected with the *M. tuberculosis* complex, who have a high risk of progression to disease, and for whom it has been determined that diagnostic testing for LTBI is warranted.

Commercially available IGRAs measure the T cell immune response to antigens that are present in *M. tuberculosis* complex but absent in *M. bovis* BCG vaccine strains and most NTM. It should be noted that although the QFT-GIT and T-Spot tests do not detect the *M. bovis* BCG vaccine strains, both assays detect the *M. tuberculosis* complex, which includes *M. bovis*. The IGRAs measure IFN-γ released by sensitized T cells incubated in the presence of *M. tuberculosis* complex-specific antigens. Until the development of the IGRAs, the TST was the only method to determine LTBI. The TST has been used for more than 100 years and has modest sensitivity and specificity. The IGRAs have specificity, especially in BCG-vaccinated populations, and are predicted to be better diagnostic tools for LTBI than the TST (250, 254–256).

For the QFT-GIT test, blood is drawn directly into a heparinized tube coated with the *M. tuberculosis* complex-specific antigens ESAT-6, CFP-10, and TB7.7. A second tube containing phytohemagglutinin (PHA) acts as a mitogen control that indicates cell functionality. A third tube, which contains no antigen or PHA, serves as a nil control. Approximately 1 ml of blood is drawn into each tube. The tubes must be incubated within 16 h of collection. After incubation for 16 to 24 h at 37°C, the plasma is collected, and the concentration of IFN-γ is determined by using an enzyme-linked immunosorbent assay (ELISA). The manufacturer provides calculations and criteria for determining a positive result. The test detects infections caused by the *M. tuberculosis* complex, which includes *M. tuberculosis*, *M. bovis*, and *M. africanum*. Specimens from patients infected with *M. kansasii*, *M. szulgai*, and *M. marinum* may respond in the assay, as these organisms have the genes encoding ESAT-6 and CFP-10 (257).

In 2015, the fourth-generation QuantiFERON-TB Gold Plus (QFT-Plus) ELISA was released in markets outside the United States. This assay also measures the response to the ESAT-6 and CFP-10 peptide antigens, but it is designed to separately measure the response of $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T helper lymphocytes to the TB antigens. The test has two TB antigen tubes: one tube contains ESAT-6 and CFP-10 peptides that are designed to stimulate responses from $CD4^+$ cells, and a second tube contains a set of peptides targeted to induce responses from $CD8^+$ cells. *M. tuberculosis*-responsive $CD8^+$ cells have been found in patients with LTBI and with active TB disease and are more frequently found in active TB disease than in LTBI (258–260). Studies have found that $CD8^+$ cell responses may be associated with recent *M. tuberculosis* complex exposure (261–263). The QFT-Plus $CD8^+$ T cell response may help distinguish active from latent TB and discriminate between recent and old infections; however, this awaits further studies. The sensitivity of QFT-Plus using culture-confirmed *M. tuberculosis* complex cases as a surrogate for LTBI is 95.3% according to the package insert (264). The specificity in persons with no known risk factors for TB is 97.6% (264).

The T-Spot assay was FDA approved in July 2008. Depending on the patient's age, 2 to 8 ml of blood is drawn into a lithium-heparin tube and processed within 30 h of collection if T-Cell Xtend is used or within 8 h if T-Cell Xtend is not used (265). Alternatively, blood may be collected into a mononuclear cell preparation tube and processed within 8 h of collection. Density gradient centrifugation is used to separate the mononuclear cells. T-Cell Xtend contains antibodies that cross-link granulocytes to red blood cells and separate the granulocytes, which may reduce the viability of mononuclear cells and reduce their ability to release interferon gamma, from the mononuclear cells during centrifugation (265). The cells are counted, and approximately 250,000 cells are added to microtiter wells that are coated with monoclonal antibodies to IFN-γ. ESAT-6 and CFP-10 antigens are then added to two wells, with a third well being used as a nil control and a fourth well containing PHA as a cell functionality control. The microtiter plates are incubated for 16 to 20 h at 37°C and then washed to remove the cells. A conjugated secondary antibody is then added, which

TABLE 16 Persons at increased risk for *M. tuberculosis* complex infection or at increased risk for progression from latent tuberculosis infection to active tuberculosis[a]

Patient population	Description
Persons at increased risk for TB infection	Those with close contact with known or suspected active TB cases
	Foreign-born persons from areas that have a high incidence of active TB
	Residents and employees in congregate settings whose clients are at an increased risk for active TB, especially if visits are frequent or prolonged
	Persons who visit areas with a high prevalence of active TB disease
	Health care workers who serve persons who are at an increased risk for active TB
	Populations defined locally as having an increased incidence of TB infection or active disease (e.g., low-income populations and persons who abuse drugs or alcohol)
	Infants, children, and adolescents exposed to adults who are at an increased risk for LTBI or active TB
Persons at increased risk for progression to active TB	Persons living with HIV
	Those who are receiving immunosuppressive therapy (e.g., TNF-α[b] antagonists, systemic corticosteroids, or immunosuppressive drug therapy following organ transplantation)
	Those who were infected with *Mycobacterium tuberculosis* within the last 2 yr
	Those with a history of untreated or inadequately treated active TB, including those with fibrotic changes on chest radiograph consistent with prior TB
	Those with silicosis; diabetes mellitus; chronic renal failure; leukemia; lymphoma; or cancer of the head, neck, or lung
	Those who have had a gastrectomy or jejunoileal bypass
	Those who weigh $<$90% of their ideal body wt
	Cigarette smokers and those who abuse drugs or alcohol
	Those defined locally as having an increased incidence of active TB, including medically underserved or low-income populations
	Infants and children aged $<$5 yr who are at an increased risk for a poor outcome (e.g., meningitis, disseminated disease, or death) if active TB occurs

[a]Adapted from reference 250.
[b]TNF-α, tumor necrosis factor alpha.

binds to the IFN-γ secreted by the T cells (and captured by the primary antibody). A substrate is then added, which produces spots where IFN-γ was secreted by the T cells. The spots that represent sensitized effector T cells are counted. For the T-Spot.TB assay, a positive test result is based on the number of spot-forming units. In the United States, the FDA-approved criterion for a positive test result is $>$8 spots, $<$4 spots are considered a negative test result, and 5 to 7 spots are considered a "borderline" result. As for all tests, the results should be interpreted in conjunction with results of other diagnostic tests and epidemiological information to help determine the *M. tuberculosis* complex infection status of the patient.

The 2010 CDC guidelines state that FDA-approved IGRAs, including QFT-G, QFT-GIT, and T-Spot, can be used similarly to the TST for the diagnosis of *M. tuberculosis* complex infection in children and adults (250); this includes persons at an increased risk for *M. tuberculosis* complex infection and those at an increased risk for progression to active TB, as listed in Table 16. Individuals in these two categories will likely benefit from treatment for LTBI. IGRAs may also be used for surveillance purposes, in contact investigations, to screen health care workers and other groups at risk for LTBI, and to identify persons who are likely to benefit from treatment. The guidelines issue caution in using IGRAs for children $<$5 years of age. Specificity is expected to be high for children, but additional studies are needed to evaluate the performance of IGRAs for this population.

The CDC guidelines also state that laboratories should report both the qualitative test result interpretation and the quantitative assay values along with the criteria used for the test result interpretation (250). Reporting the quantitative value is important, especially when interpreting results from serial testing (e.g., health care workers), when IFN-γ levels are close to the cutoff value separating positive and negative results. Several studies have shown that in individuals serially tested with QFT-IT, those with quantitative values near the cutoff of 0.35 IU/ml are more likely to have variability in their qualitative result interpretation, positive or negative, than individuals with quan-

titative results farther from the cutoff value (212–214). Providing this information will allow the health care provider to make a better assessment of the IGRA results.

There are both advantages and disadvantages to consider when implementing IGRAs. Unlike the TST, IGRA results can be available within 24 h, if performed on-site, without the need for a second patient visit. In TB contact investigations, a significant number of individuals do not return to have their skin tests read, and results are not available to help public health professionals prioritize follow-up (266). In addition, errors in the placement and reading of skin tests can result in misinterpretation of the results and inappropriate patient management. Disadvantages of IGRAs include the need to draw blood, stringent blood collection and mixing requirements, and time limitations for initial specimen processing or incubation. The T-Spot Xtend reagent can be used to extend the time from collection to sample processing in the laboratory to 30 h, as noted above. However, this still may not be enough time to transport the specimen to a distant reference or public health laboratory. IGRAs are complex assays. Compared to the TST, which requires 5 steps to complete, QFT-GIT requires at least 126 separate steps for one test result (267). The cost of IGRAs is higher than that of the TST, but studies have shown that the lower rates of positivity of IGRAs and the advantage of a single patient encounter, which provides results for a higher percentage of patients, may lead to overall savings to the health care system (268).

IGRAs have the potential to improve the diagnosis and management of TB; however, there are still outstanding issues concerning the performance characteristics of the assays, interpretation of the results, and limitations of IGRAs that require further research. For example, although quantitative results are useful for evaluating the likelihood of conversions or reversions when values are close to the assay cutoff value, there is no consensus on the interpretation and significance of IGRA conversions and reversions at this time. Quantitative IGRA results have not been shown to have prognostic value, and it is still not known if IGRAs are better than the TST in predicting progression to active TB.

Several factors have been shown to impact the results of IGRAs (206–211, 269–274). Preanalytical factors such as the amount of time from blood collection to incubation can significantly affect IGRA results (269). Other studies have shown that the time of day when blood is drawn can also influence the IGRA result (270). A study by Whitworth et al. showed that different incubation times within the parameters of the assay protocols can affect the quantitative results and the interpretation of the results of the assays (271). Another study showed variability of results related to how the QuantiFERON tubes are shaken and the quantity of blood in each tube (275). It is important for laboratories to standardize and control the parameters of the assays to optimize reproducibility.

Health care workers are at an increased risk of exposure to *M. tuberculosis* complex bacteria and have traditionally been screened for LTBI by using the TST. Two excellent systematic reviews have summarized data from the studies that have evaluated the use of IGRAs on health care workers (276, 277). Some studies have shown high rates of IGRA conversions that are not consistent with the low rates of TB seen in the United States and the exposure risks of health care workers (278). In 2016, Gamsky et al. (279) reported a study on the use of serial QuantiFERON-TB IGRAs on emergency responders in a low-TB-prevalence county in California from 2001 to 2013. Those researchers concluded that the QuantiFERON-TB IGRA should not be used for the diagnosis of LTBI in low-risk populations because of frequent and irreproducible positive results. Most studies have also shown high rates of reversions, which are more likely to be seen among those with IFN-γ values or spot counts near the diagnostic cutoff (278). Some health care facilities have established their own cutoffs or have implemented retesting strategies to eliminate false-positive conversions (280, 281). A 2015 study by King et al. that included over 19,000 health care workers from 19 U.S. hospitals showed positivity and conversion rates consistent with known TB risk factors using the T-Spot.TB assay (282). Suggestions for the standardization of occupational testing programs have been proposed to limit the variability in test results and interpretations (283).

Because of the complexity of IGRAs and the many factors that can impact the results of the assays, it is especially important to have a strong quality assurance program in place. Key quality assurance monitors should be identified, such as the percentage of positive results; the percentage of indeterminate or invalid results; the distribution of IGRA values, especially those near the cutoff value; and correlation of positive results with the patients' risk factors for TB infection. Gaur et al. found that the blood volume and tube shaking impact IGRA results (275). In many laboratories, IGRAs are performed in the serology department and not in the TB laboratory. The personnel performing the testing must establish a close relationship with the state TB program, local public health programs, infection control programs, employee health programs, and health care providers to determine the correlation of IGRA results with clinical presentation, chest radiography results, results of other diagnostic tests, and epidemiological risk factors for TB of the patients. It is important for the laboratory to educate health care providers about the variability and limitations of IGRAs so that the results can be interpreted appropriately.

USING A REFERENCE LABORATORY

The need for, and availability of, diagnostic mycobacteriology services has undergone substantial changes in the recent past. These changes are due to (i) the overall decreased incidence and prevalence of TB over the decades, with a concurrent increase in the isolation of NTM; (ii) the fact that mycobacterial infections vary geographically and by patient populations; (iii) the fact that many community hospitals and clinics see few or no patients with NTM infections; (iv) the centralization of clinical services that provide care for patients with mycobacterial infections (e.g., public health clinics); (v) the increasing complexity and sophistication of testing required to provide diagnostic mycobacteriology services; (vi) the need to closely link clinical and laboratory services as part of integrated systems for caring for patients with mycobacterial infections; and (vii) the ongoing emphasis on controlling costs in health care. Not surprisingly, many clinical laboratories have opted to discontinue offering a full range of diagnostic mycobacteriology services, instead referring parts or all of these services to reference laboratories.

When To Refer Specimens/Isolates to a Reference Laboratory

Clinical microbiology laboratories are faced with decisions as to which laboratory services to offer in-house versus which ones to send to a reference laboratory (103). Factors that must be considered include the scope of clinical services provided by the hospital or clinics served by each laboratory, test volumes, the technical capacity of the laboratory, whether the laboratory is part of a larger health care system or network, and the availability of reference laboratory services. For most hospital-based clinical microbiology laboratories, providing a full scope of mycobacterial cultures and susceptibility testing is neither necessary nor appropriate. However, almost all hospital-based laboratories should offer AFB smears.

A more contemporary decision is whether to offer rapid diagnostic testing by methods such as the Xpert assay or a line probe assay such as the GenoType MTBDR*plus* assay. This is a decision not only for laboratories that perform both AFB smears and cultures, where rapid assays must be integrated into a traditional testing approach, but also for laboratories that may need access to a rapid diagnostic test in urgent situations. The Xpert assay recently received an expanded product claim that allows clinicians to remove patients with suspected TB from AII based on negative test results (147). Because all rapid assays are more sensitive when testing AFB smear-positive specimens, the results of rapid molecular assays must be interpreted in conjunction with the results of AFB smears (even when AFB smears are not performed on-site). Although the Joint Commission standard for reported AFB smear results does not mention this test, it is unlikely that the standard would be interpreted differently. Therefore, optimally, Xpert results should be available within 24 h if the test is being used as part of a program to remove patients from AII based on laboratory

test results. There is no simple, straightforward algorithm for determining the scope of mycobacteriology services to offer in each laboratory.

How To Assess Reference Laboratories

Reference laboratories vary widely in their scope of services, expertise, quality, cost, test turnaround time, and use of information technology. Even though selection often may be driven by other factors, such as participation in a purchasing consortium, important factors in selecting a reference laboratory are the scope of services, expertise, and an outstanding track record in quality management systems.

Reference laboratories should provide referring laboratories with complete and explicit instructions for specimen storage and transport, including information on suitable media and temperatures and the acceptable time limit after which specimens are no longer suitable for culture or other testing. Information should be provided for both clinical specimens as well as cultures that need further testing, with specific instructions for different specimen types as well as any need for special culture conditions (e.g., temperature of incubation). Because of the potential for the loss of microbial recovery due to mishandling or delays, laboratories should monitor recovery rates and any discrepant results carefully as part of a quality assurance program.

The turnaround time should also be monitored as part of the laboratory's quality assurance program. Part of the evaluation and selection of competing reference laboratories should be an analysis of their total turnaround time from specimen receipt to reporting results to clients. Cost is another obvious factor in selecting reference laboratories, but due to the wide variability in contracts and other factors, it often is beyond the control of the clinical laboratory director. Moreover, large-volume reference laboratories typically are quite competitive when it comes to cost. The use of information technology is of increasing importance due to the rapid expansion of the use of integrated electronic health records. From a laboratory perspective, systems such as these function at their full potential only when used for test ordering, result reporting, and quality assessment. Moreover, because of their high cost, they are cost-effective when used for automated orders, reporting, and billing. In other words, manual test ordering and reporting are not congruent with the use of these systems. A significant part of the use of these systems is the ability to develop interfaces with reference laboratories so that test ordering and reporting are done via direct interfaces or through a middleware system.

It is apparent that many factors must be taken into consideration when selecting a reference laboratory (284) and that it is not an easy or straightforward decision, but consensus-based standards for assessing and selecting reference laboratories are now available (285).

PUBLIC HEALTH REQUIREMENTS

The laboratory community plays a critical role in the public health system and its efforts to control and eliminate TB in the United States. Each laboratory (hospital, commercial, reference, and public health), along with health care providers, plays integral roles in the treatment and management of TB patients and their contacts. The sooner a suspected TB case is reported to public health and TB control programs, the fewer transmissions and subsequent TB cases there will be. Public health reporting requirements vary widely from state to state. It is incumbent on the clinical laboratories to be aware of the TB reporting requirements (statutes, administrative code, and regulations) in their jurisdiction.

All 50 U.S. states and the District of Columbia have reporting requirements for TB disease. Eighty-five percent (44/52) of jurisdictions require reporting of positive AFB smears. Fifty-three percent (28/52) of jurisdictions require reporting of a positive TB culture within 24 h or within 1 business day of identification. The reported time frame for clinical reporting varies from immediately to up to 7 days or weekly. Twenty-three percent (12/52) of jurisdictions require reporting of phenotypic AST results. However, no jurisdiction currently requires reporting of molecular drug resistance results. Sixty-

TABLE 17 Laboratory information that may be reportable to public health programs[a]

Receipt of an order for a TB culture
Receipt of an order for a TB NAAT
Positive TB NAAT result
Positive AFB smear
Culture positive for suspected or confirmed *Mycobacterium tuberculosis* complex organism
Culture positive for NTM
Positive test for LTBI (positive TST or IGRA result)
Molecular and phenotypic AST results

[a]TB, tuberculosis; NAAT, nucleic acid amplification test; AFB, acid-fast bacilli; NTM, nontuberculous mycobacteria; LTBI, latent tuberculosis infection; TST, tuberculin skin test; IGRA, interferon gamma release assay; AST, antimicrobial susceptibility testing.

nine percent (36/52) of jurisdictions require submission of the TB isolate to a local or state public health laboratory for confirmation testing and submission to the national genotyping program. Electronic reporting is currently mandatory for 6 jurisdictions. Reporting of LTBI is required by 23% (12/52) of jurisdictions, some only for children <5 years of age. Twenty-five percent (13/52) of jurisdictions require reporting of NTM (57).

Recently, researchers from the Oregon Health and Sciences University and the Oregon Health Authority described their experience with extrapulmonary NTM reporting. They also discussed possibilities of monitoring trends in pulmonary NTM disease either by mandatory reporting or by alternative methods, such as voluntary reporting of pulmonary isolates by laboratories supplemented by intermittent surveys and special surveillance projects (286).

Table 17 shows information/results that may require reporting to public health programs, which vary from state to state. If specimens are sent to a reference laboratory for testing, the referring laboratory is responsible for reporting the results to public health programs unless the reference laboratory has agreed to do the required reporting. For example, California mandates that if the referral laboratory is out of state, the California laboratory that receives the report is responsible for reporting the results to the public health system (287).

Additionally, there are various time requirements for reporting among public health departments. For example, in Wisconsin, the identification of a *M. tuberculosis* complex isolate from a culture or a positive *M. tuberculosis* complex NAAT result must be reported immediately to the public health program with a follow-up written report within 24 h (288); Nebraska requires reporting within 7 days of detection or diagnosis (289). Electronic laboratory reporting has been implemented in many jurisdictions and has increased the timeliness and efficiency of reporting to public health systems and decreased the burden on the laboratory.

Another important public health responsibility of the clinical laboratory is the submission of initial isolates from culture-confirmed TB patients to the public health laboratory for genotyping by the CDC Tuberculosis Genotyping Program (290). This program provides funding and support for universal genotyping with the goal of typing all *M. tuberculosis* complex culture-positive cases in the United States. The methods routinely used, as part of this program, include spoligotyping and mycobacterial interspersed repetitive unit (MIRU) analysis. If two TB patients have isolates with different genotypes, this indicates that the patients are not likely involved in the same chain of transmission. Genotyping, combined with epidemiological data, helps identify TB patients involved in recent transmissions, is a method for monitoring epidemiological trends, and has significant impacts on TB control. Genotyping enables the earlier detection of outbreaks, resulting in more rapid responses and fewer transmissions. It may also identify TB cases with undetected epidemiological links and in unusual settings, including outbreaks that occur in patients residing in different jurisdictions. For the laboratory, genotyping identifies false-positive cultures that may be the result

of mislabeling of specimens, cross-contamination of cultures, or other problems (291). Ultimately, the timely submission of isolates to public health laboratories aids in the control of TB in the United States.

RESOURCE-LIMITED SETTINGS

TB surpassed HIV as the leading cause of death from infectious disease in the world in 2014 (15). Pulmonary TB is of great public concern, so not surprisingly, this is the sole or primary focus of mycobacterial diagnostics in resource-limited settings. Because many resource-limited settings continue to lack access to quality mycobacterial diagnostic testing, and because of continuing urgent public health concerns for controlling TB, expansion of access to and the capacity for mycobacterial testing should continue to emphasize TB.

The diagnosis and treatment of TB have a long history of being integrated with public health control programs. Thus, diagnostic testing should be aligned and appropriate for the capacity for patient evaluation and treatment in each location. AFB smears remain a mainstay of diagnosis because they provide rapid preliminary diagnostic information and a rough assessment of infectiousness and guide interpretation of the results of rapid molecular diagnostic tests. In rural outpatient clinics, AFB smears may be the only practicable diagnostic test. As clinics become larger and have access to improved diagnostic testing, the introduction of rapid diagnostic tests such as line probe assays or the Xpert assay becomes feasible, although several factors come into play when deciding at which point these tests can be used in a cost-effective manner. In hospitals in larger cities, mycobacterial cultures become a practical approach, although many hospitals do not have the capacity to perform AST. Finally, teaching hospitals, large private hospitals, and national public health laboratories often have the capacity to provide the full scope of mycobacterial diagnostic testing.

The WHO has endorsed a number of diagnostic tests for TB control programs, including light-emitting diode (LED) microscopy (292), line probe assays (293, 294), and the Xpert assay (295), and more are under development. On a strict market basis, none of these tests would be affordable in most resource-limited areas, and thus, the WHO has developed partnerships with several governmental and nongovernmental organizations to reduce the acquisition cost to affordable levels. Unlike rapid diagnostic tests for malaria, for which there are dozens of products and manufacturers (296), only a few diagnostic tests for TB have been developed (297), thereby providing more standardized products, simpler supply chains, easier quality control programs, and better evaluation of the effectiveness of the products in TB control programs. In Spring 2016, the WHO updated its policy on drug-resistant TB treatment (298), allowing the use of a 7-drug regimen for only 9 to 12 months, instead of the 18- to 24-month regimen. To use this shorter MDR-TB regimen, drug resistance to second-line drugs needs to be excluded. In patients with confirmed RIF-resistant TB or MDR-TB, the Genotype MTBDR*sl* assay may be used as an initial direct test, over phenotypic culture-based AST, to detect resistance to FQ and to the second-line injectable drugs (299).

It is recognized that a detailed description of providing access to mycobacteriology services in resource-limited countries is beyond the scope of this review. For such services to be effective, they must be integrated with systems for clinical care and public health programs, in part because they focus primarily on TB and not on other mycobacteria but also because the detection of drug resistance is of paramount concern. Rapid test turnaround times are also a critical issue because many patients in these settings are from rural areas and have only limited access to health care facilities (300).

CONCLUSION

In 1994, Salfinger and Pfyffer (301) published a review article entitled *The New Diagnostic Mycobacteriology Laboratory*, describing a brief comparison between con-

temporary traditional methods and the latest developments for the direct detection of AFB. They also stressed that if patient care and public health are always considered paramount, regardless of admission time or hospital type, etc., the concept of services at that time had several shortcomings. They proposed that one way to manage those challenges was to sort and allocate specimens per a system of priorities. A model Fast Track program for tuberculosis was created to form a network of laboratories to expedite testing for highly infectious TB suspects (302). With the establishment of such a network, when new assays are validated and implemented in the central laboratory, the entire network of enrolled submitting entities immediately benefits. Similar approaches were initiated in the United Kingdom (303) and in Portugal (304). There was a growing realization that no single method by itself is sufficient to address the entire spectrum of diagnostic challenges. To streamline the best choice for laboratory diagnosis and patient management, a centerpiece of this practice guideline is the ideal algorithm and alternative algorithms for testing specimens for mycobacterial diseases by utilizing this concept.

In the past 25 years, a tremendous change in the epidemiology of drug-resistant TB and pulmonary NTM has occurred, thus warranting even further shortening of turnaround times. By the same token, molecular diagnostic assays are becoming the standard of care. With the plethora of tests available, ongoing communication between the health care provider and the laboratory is essential. In this review, we provide an in-depth discussion of the latest developments in mycobacteriology testing as well as a holistic approach to sample collection, reporting of laboratory results, and interpretation of results for the health care provider.

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