Recent Advances in the Laboratory Detection of Mycobacterium tuberculosis Complex and Drug Resistance

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The global control of tuberculosis remains a challenge from the standpoint of diagnosis, detection of drug resistance, and treatment. Because treatment can only be initiated when infection is detected and is based on the results of antimicrobial susceptibility testing, there recently has been a marked increase in the development and testing of novel assays designed to detect Mycobacterium tuberculosis complex, with or without simultaneous detection of resistance to isoniazid and/or rifampin. Both nonmolecular and molecular assays have been developed. To a large extent, the nonmolecular methods are refinements or modifications of conventional methods, with the primary goal of providing more-rapid test results. Evaluations of molecular assays have generally shown that these assays have variable sensitivity for detecting the presence of M. tuberculosis complex and, in particular, are insensitive when used with smear-negative specimens; high sensitivity for detecting resistance to rifampin; and variable sensitivity for detecting resistance to isoniazid.

Infections caused by Mycobacterium tuberculosis complex remain one of the most important global public health issues: there were 9.4 million cases of tuberculosis (TB) in 2009, causing 1.7 million deaths [1]. Of these, 1.1 million cases and 380,000 deaths occurred in persons infected with HIV [1]. During 2008, there were an estimated 440,000 cases of multidrug-resistant TB (MDR-TB), or 3.3% of all new cases of TB, resulting in 150,000 deaths [1]. Extensively drug-resistant TB (XDR-TB) has now been confirmed in 58 countries [1]. Estimated TB incidence rates are highest in sub-Saharan Africa and in Southeast Asia, regions that also have high HIV infection rates and inadequate access to health care in many areas. For global TB control programs to be effective, particularly in these regions, improved diagnostic methods are needed. Access to improved TB diagnostics is of particular importance in areas where patients have infrequent or intermittent access to health care and sites where providers are not able to wait for results from reference laboratories before either withholding or initiating anti-TB therapy.

Despite the need for better diagnostic tests, until recently, there has been little emphasis on developing new tests for the diagnosis of TB. From a global perspective, many laboratories use the same methods today that were in use nearly half a century ago: conventional stains such as Ziehl-Neelsen or Kinyoun for staining sputum smears, egg-based media for culture, and solid media for antimicrobial susceptibility testing (AST). Although it is now more common for laboratories to use fluorochrome stains to stain smears and liquid-based media for cultures, these methods are not widely used in small hospitals or clinics because of the need for greater technical expertise and equipment. Too many laboratories around the world do not have access to these methods. Antimicrobial susceptibility testing is even more problematic, because it is difficult to do well, the turn-around time is often measured in months, some drugs often show discordant results (particularly ethambutol), and AST for...
second-line drugs remains poorly standardized and not widely available. Thus, there is a pressing need for new methods that will allow for both the rapid detection of TB in patients and for AST to identify patients who are infected with resistant strains.

To address these issues, a number of efforts are under way to develop new methods for the diagnosis of TB and the detection of drug resistance, including both nonmolecular and molecular methods. This update will describe some of these methods and the practical limitations of using these assays in the field.

**NONMOLECULAR METHODS**

A number of nonmolecular methods for detecting *M. tuberculosis* complex and/or antimicrobial drug resistance have been developed. A few of these methods are designed to improve existing technology, such as specimen processing and smears, whereas others are based on newer technologies. The approach of improving existing methods, rather than developing new methods, has much going for it. First, smear microscopy is the most widely used method for the diagnosis of TB, and as a result, there exists a widespread infrastructure for performing this test. Second, introducing these methods would require fewer resources than would introducing new technologies. Third, these methods are likely to be less expensive than new technologies. Lastly, health care providers are familiar with these methods, how to interpret results, and the strengths and limitations of the methods. Nonetheless, smear microscopy is an insensitive test method that, despite decades of training and infrastructure development, has not been effective in helping control TB. Antimicrobial susceptibility testing is even more problematic, because it is not available in many areas where it is needed.

**Fluorescent Light Emitting Diode (LED) Microscopy**

Because of the limitations of conventional light microscopy using stains such as Ziehl-Neelsen, fluorochrome stains such as auramine were introduced that improve the sensitivity of the test and take less time to perform. However, fluorescence microscopy has the limitations of requiring a fluorescent microscope, a dark room, and an expensive light source [2]. Mercury vapor light sources used for this type of microscopy can also pose a hazard if bulbs are broken. To overcome these limitations, LED microscopy was developed. This type of microscopy uses LED technology as a light source but still allows for the advantages of using a fluorescent stain while eliminating most of the disadvantages of fluorescence microscopy [2]. LED microscopy is more sensitive and equally specific, compared with either conventional light or fluorescent microscopy [2]. The World Health Organization (WHO) recommends that conventional fluorescence microscopy be replaced by LED microscopy, and that LED microscopy should be “phased in as an alternative for conventional light microscopy” [2].

**Microscopic Observation Drug Susceptibility (MODS) Assay**

The MODS assay is a broth microtiter method designed to detect *M. tuberculosis* complex and to detect resistance to isoniazid and rifampin [3–7]. The method uses standard microtiter plates and other materials that are readily available in larger diagnostic laboratories. The method is straightforward: microtiter plates are prepared that contain Middlebrook 7H9 broth medium, growth supplements, and antimicrobial agents to prevent overgrowth of bacterial contaminants. Anti-TB drugs, at different concentrations, are added to some of the wells. The wells are inoculated with the clinical specimens, sealed to prevent contamination, and examined periodically for growth. Cultures positive for pathogens show growth with coring; *M. tuberculosis* complex is distinguished from other mycobacteria that exhibit coring, such as *Mycobacterium chelonae*, by the more rapid growth of the latter (although in some studies, more rapid growth of *M. tuberculosis* has been reported, which could potentially lead to misidentification of an isolate as *M. chelonae*). Detection of drug resistance is by inhibition of growth in wells containing drugs. Because mycobacteria grow faster in liquid than on solid media, detection of tubercle bacilli can occur more quickly than with other culture methods. In the same way, inhibition of growth by anti-TB agents allows for the rapid detection of drug resistance.

Although the MODS assay is inexpensive and simple, it is best suited for larger laboratories that already have an existing infrastructure for TB diagnostic testing. The method requires training and technical expertise, the ability to perform testing at Biosafety Level 3, and equipment (such as a stereoscopic microscope), reagents, and supplies that may not be available in smaller laboratories. The method is not yet standardized.

The performance characteristics of the MODS assay were summarized in a recent meta-analysis [8]. For detecting low-level resistance to isoniazid the pooled sensitivity of the assay is 97.7% and specificity is 95.8%. For detecting high-level isoniazid resistance, the sensitivity decreases to 90.0%, but the specificity increases to 98.6%. For detection of rifampin resistance, the pooled sensitivity is 98.0% and the specificity is 99.4%. This meta-analysis did not summarize the ability of the assay to identify the presence of *M. tuberculosis* in sputum specimens. The published sensitivity of the assay varies from 87.4% to 97.8%, although the assay was compared with different gold standards in these studies [3–5, 7]. The contamination rate for the MODS assay, although lower than that of solid media, is higher than that of liquid media [8].

**Colorimetric Assays**

A colorimetric method for detecting microbial growth in drug-resistant strains was described in 1998 and subsequently
evaluated in a limited number of clinical trials [9–14]. The assay is based on the observation that growing tubercle bacilli convert a yellow dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT] to a purple color that can be detected visually or by use of a spectrophotometer. In field trials, the method has been shown to have a high degree of concordance with conventional AST [11–14]. The method has been compared with a nitrate reduction assay and a resazurin assay for detecting resistance to isoniazid, rifampin, ethambutol, and streptomycin; similar results were obtained for isoniazid and rifampin, but only the nitrate reduction assay showed a high level of concordance with all of the first-line drugs [14]. Although these methods are conceptually straightforward, they are likely to be useful primarily in larger laboratories with the capacity to perform more complex assays.

**MDR-XDR TB Color Test**

This assay is based on Thin-Layer Agar (TLA) technology, with both culture and direct AST method on a single agar plate. This particular TLA assay is based on color changes in the 4 quadrants of the plate, with 1 quadrant for detection of growth and the other 3 quadrants for AST (1 quadrant each for isoniazid, rifampin, and ciprofloxacin). There are only limited published data on TAL assays as a group [8, 15–17] and no published data regarding the performance characteristics of the MDR-XDR TB assay. The available data suggest that TLA assays have high sensitivity and specificity for detecting drug resistance [8, 15–17] and that the contamination rate for TLA assays appears to be much lower than that of either solid or liquid media [8].

**Other Nonmolecular Methods**

Broadly speaking, this last group of methods is based on incremental changes of standard test methods and is intended to modify existing methods to increase their availability for laboratories with limited resources and to improve turn-around time for test results [18–23]. Field trials of these methods have yielded variable results. For example, one method, the Universal Sample Processing method, was shown to have a higher sensitivity for smear microscopy, compared with the standard n-acetyl-l-cysteine method, to improve the detection of acid-fast bacilli so that smear categories (ie, ≥1, ≥2, and ≥3) were higher, and to decrease contamination rates [21]. In a subsequent evaluation, however, mycobacterial culture results were found to more often be negative with the USP method, there was no difference in contamination rates between the 2 methods, and the sensitivity of smears was not significantly different [22]. Overall, evaluations of these various methods have shown improvements in detection of mycobacteria, but what is needed next are clinical trials directly comparing each method to see which is best and cost-benefit analyses of each method.

**MOLECULAR METHODS**

A number of molecular assays have been designed to detect the presence of *M. tuberculosis* and to detect resistance to isoniazid and/or rifampin. The potential advantages of molecular assays are the ability to (1) design assays that are highly sensitive and specific; (2) manufacture some assays in large quantities, allowing for decreased cost and ease of standardization in field use; (3) yield rapid results; and (4) be used more widely, because they require less training and infrastructure than do conventional mycobacterial cultures and AST. These potential advantages must be weighed against the disadvantages of these assays, some of which are common to all molecular assays and others specific to particular assays. Among the disadvantages of molecular assays are (1) a need for laboratory infrastructure that can accommodate molecular testing, (2) cost, (3) a continued need for cultures for AST, and (4) most work better with smear-positive than with smear-negative specimens.

**Line-Probe Assays**

This technology involves a series of steps including extraction of DNA from mycobacterial isolates or directly from clinical specimens, polymerase chain reaction (PCR) amplification of nucleic acid sequences, hybridization of labeled PCR products with oligonucleotide probes immobilized on a strip, and colorimetric development that allows for lines to be seen where the probes are located (hence, the term “line-probe” assay) [24]. In 2008, the WHO issued a policy statement regarding the molecular line-probe assays for use in detection on *M. tuberculosis* and for detection of drug resistance [24]. This document describes the technology and necessary infrastructure for performing the test and makes policy recommendations regarding use of the test [24].

The first line-probe assay was the INNO-LiPA Rif TB (Innogenetics NV) [25–29]. The results of clinical evaluations of the assay indicated that it accurately detects resistance to rifampin, but some of the evaluations showed that the assay was less sensitive for the detection of *M. tuberculosis* complex [25–28]. A meta-analysis performed in 2005 showed that 12 of 14 published studies showed a sensitivity ≥95% with a specificity of 100% but that, in studies in which the assay was applied to clinical specimens, the sensitivity ranged from 80% to 100% [28]. One study showed that the assay could be used successfully in a resource-poor setting, compared with a reference laboratory [29].

The second line-probe assay was the GenoType MTBDR (Hain Lifescience) [30–39]. This assay was originally developed as the GenoType MTBDR assay, but early evaluations showed that the assay did not detect drug resistance to a satisfactory degree, detecting only 90%–95% of isolates with rifampin or low-level isoniazid resistance [30–32]. The assay was eventually
modified to include detection of more rpoB and inhA mutations, with the name GenoType MTBDRplus [33–36]. Although 2 evaluations of the new assay showed improvement of the detection of isoniazid resistance [33, 34], 3 other evaluations showed that detection of isoniazid resistance remained sub-optimal (particularly for strains with low-level resistance) [35–37]. A meta-analysis performed in 2008 confirmed these findings; the assay shows high sensitivity and specificity for detecting resistance to rifampin but variable results for detecting resistance to isoniazid [38]. A second meta-analysis performed the subsequent year showed similar results, although in this analysis, the pooled sensitivity of the GenoType MTBDRplus assay showed better sensitivity for detecting isoniazid resistance [39]. Overall, results of these evaluations indicate that the assay is of limited use with smear-negative specimens and that detection of isoniazid resistance is more variable but generally lower than detection of rifampin resistance.

Another version, GenoType MTBDRsl, is designed to detect resistance to fluoroquinolones, ethambutol, kanamycin, amikacin, and capreomycin [40, 41]. Two evaluations of this assay have shown promising but variable results for detection of resistance to the second-line drugs [40, 41].

**Loop-Mediated Isothermal Amplification**
The Loop-Mediated Isothermal Amplification assay (Eiken Chemical Company) relies on a novel form of nucleic acid amplification with sufficient efficiency that enough DNA is generated to enable detection by visual inspection of fluorescence [42]. The method has been evaluated on a limited basis and has been shown to have high sensitivity for smear-positive specimens but low sensitivity for smear-negative specimens [42].

**Oligonucleotide Microarray**
Oligonucleotide microarray technology allows for the simultaneous detection of multiple genetic sequences, which can be used to detect either conserved sequences for detection of microorganisms and/or detection of mutations in sequences that confer drug resistance of an isolate. One of these assays, The TB-Biochip (Engelhardt Institute of Molecular Biology), has been validated for the ability of the system to detect rifampin resistance in *M. tuberculosis* [43]. In a small study comparing the microarray with conventional AST, the assay showed a sensitivity of 80% for detecting rifampin resistance [43].

**Xpert MTB/RIF**
The Xpert MTB/RIF assay (Cepheid) is a self-enclosed, rapid PCR device that, to some extent, mitigates many of the limitations of other molecular assays [44–46]. This is largely because the device is self-enclosed and, therefore, requires less sophisticated infrastructure in terms of laboratory facilities, user training, and supply chain management. In a limited evaluation, the assay was shown to be 100% sensitive for detecting smear-positive isolates but only 71.7% sensitive for detecting smear-negative culture-positive isolates [45]. In a larger field trial, the assay was shown to be 98.2% sensitive for the identification of culture-positive isolates but only 72.5% sensitive for the identification of smear-negative culture-positive isolates; the test had a reported specificity of 99.2% [46]. In this study, for isolates who were smear-negative but culture-positive, by adding a second MTB/RIF test increased the sensitivity by 12.6%; adding a third MTB/RIF test increased the sensitivity by an additional 5.1% (for a total sensitivity of 90.2%) [46]. In the same study, the assay was shown to be highly sensitive for detecting rifampin resistance, correctly identifying 97.6% of rifampin-resistant isolates and 98.1% of rifampin-susceptible isolates. One obvious disadvantage to this system is the inability to test for and detect isoniazid resistance. Other potential disadvantages include cost and, although to a lesser extent than line-probe assays, a continued need for adequate laboratory infrastructure and training of personnel [46]. The WHO has endorsed use of this assay [47].

**PRACTICAL LIMITATIONS OF RAPID DIAGNOSTIC ASSAYS**

Despite their rapid turn-around time, comparatively low cost per test result, and apparent simplicity of use, rapid diagnostic assays have important limitations. First, none of the assays eliminates the need for mycobacterial cultures. Second, many molecular methods still require laboratory facilities, an adequate water supply, supply chain systems, and technical expertise lacking in the very places where the assays are needed. Third, many molecular assays work well only with smear-positive specimens, which is most problematic in sub-Saharan Africa, where many HIV-infected patients with TB are smear negative. Lastly, the reported cost of the assays, although relatively low, is offset to some extent by the need for adequate infrastructure. Because of these limitations, use of these assays in the field is likely to be limited to those areas where governments or other health programs provide sufficient funding to develop and sustain a necessary infrastructure. As a result, it is likely that these assays will not be used widely in rural clinics and hospitals. Coordination of the development and use of new tests for the diagnosis of TB should be of high priority.

Another important consideration in the use of rapid tests is whether the shortened test turn-around time will have an important effect on the outcome of patient care. As noted in an evaluation of TB treatment in Peru, test turn-around time is only one component in the evaluation and treatment of patients with TB [48]. Because many other factors affect the overall time to evaluate and treat patients, incremental decreases in test turn-around time may not have the desired impact unless changes are made in the overall process of patient care [48].
SUMMARY

A number of new methods and assays have been developed for the detection of *M. tuberculosis*, with or without detection of drug resistance. However, rapid methods are not a replacement for culture, most are not reliable when used with smear-negative specimens; conventional AST is still needed to confirm cases of XDR-TB; to test for resistance to drugs other than isoniazid and rifampin, none of the methods detect all resistant strains; and in many parts of the world, the existing infrastructure is inadequate for these assays to be used on a widespread basis. Moreover, it is not yet clear that use of these assays, without other changes in overall diagnosis and treatment programs, will have the effect on TB control that is needed in many areas.

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References


