Facing the Crisis: Improving the Diagnosis of Tuberculosis in the HIV Era

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Although the human immunodeficiency virus (HIV) infection pandemic has had a catastrophic impact on tuberculosis (TB) control efforts, especially in sub-Saharan Africa, most of the fundamental concepts reflected in the directly observed treatment, short course (DOTS) strategy still hold true in the HIV era. What has changed, and dramatically, is the importance of speedy and accurate TB diagnosis and the difficulty of achieving this. The disproportionate amount of smear-negative disease in sub-Saharan Africa, which shoulders two-thirds of the global burden of HIV infection and acquired immunodeficiency syndrome, has greatly complicated TB case detection and disease control. Now, 15 years after TB rates began to soar in countries where HIV infection is prevalent, we have learned that the conventional approach—passively waiting for patients with advanced symptomatic disease to make their way to microscopy centers for diagnosis—has disastrous consequences. Without better diagnostic tools for TB and effective strategies for their implementation, transmission will not be interrupted, mortality will not be checked, and TB will not be controlled in areas where HIV infection is prevalent. Fortunately, a number of technical opportunities exist for the creation of improved diagnostic tests. Developing and exploiting such tests to support TB control in HIV-infected populations is an urgent priority. A substantial public sector effort is under way to work in partnership with the biotechnology industry to accelerate progress toward that goal. In this article, we will define the need for better TB tests and describe technologies being developed to meet that need.

BACKGROUND

HIV-mediated immunosuppression impairs granuloma formation, resulting in both ineffective containment of Mycobacterium tuberculosis bacilli and diminished formation of pulmonary cavities [1, 2]. These effects manifest clinically as frequent extrapulmonary disease [3], atypical chest radiographic findings [4, 5], greater involvement of the lower lobes of the lung, and lower concentrations of bacteria in sputum [6].

Since Koch’s discovery of tuberculous bacilli in 1882, microscopic detection of the bacilli in clinical specimens has remained the cornerstone of tuberculosis (TB) diagnosis in low- and middle-income countries. The failure to control TB in HIV-endemic areas has underscored 2 major limitations to microscopic diagnosis: the low clinical sensitivity of the technique in HIV-infected individuals and the logistic difficulty of ensuring good access to quality microscopy in resource-limited settings.

The first problem is sensitivity. In research settings in which culture comparison is used, the average sensitivity of sputum microscopy for the detection of pulmonary TB is <60% in immunocompetent populations [6–15] and is substantially lower among people infected with HIV [16–19]. The actual sensitivity of microscopy under field conditions may be much lower. The fraction of HIV-coinfected individuals with pulmonary TB that can be detected by microscopy varies widely with the degree of immunosuppression, the length of TB illness, and the local diagnostics setting, including the organization and strength of the TB control program and its laboratory infrastructure.
The second problem is limited access to quality microscopy services. The inherent low sensitivity of the test is too often exacerbated by the poor conditions under which it is performed. In overworked, underfunded laboratories, especially in areas where HIV infection is prevalent, the proportion of cases detected by microscopy is often as low as 20%–35% [20–23]. Duplicate or triplicate sputum examinations employed to help overcome this problem—each of which requires sputum collection, smear preparation, staining, and meticulous examination—delays results, and a relatively large number of patients do not complete testing or are lost to the health care system despite having tested positive for TB [24].

Limited access to diagnostic services results in substantial diagnostic delay, and patients in many countries with a high TB burden do not receive diagnoses for 3–6 months [25, 26]. This delay fuels disease transmission and increases the severity of disease when it is finally discovered. This latter fact paradoxically drives up the fraction of all patients with TB reported as having smear-positive disease, which may be misinterpreted as evidence of good performance of the microscopy network when, in fact, it represents late detection and underdiagnosis of smear-negative disease.

Frequent smear-negative disease exacerbates the difficulty of detecting HIV-associated TB, leading to additional delays while diagnostic testing or antibiotic treatment trials are being performed. For example, more than a third of patients with smear-negative TB in Malawi needed 6 visits to a health center before therapy was initiated [27]. Examination of as many as 9 sputum smears is recommended before reaching a diagnosis of smear-negative TB in many countries [28]. The failure to rapidly detect TB in immunocompromised populations has important implications both for patient care and disease control. Although the conventional wisdom is that smear-negative cases do not contribute significantly to transmission, it is not known whether this holds true in populations in which HIV-associated immunosuppression is common. What is clear is that the failure to detect TB early in HIV-infected individuals is lethal. Up to 20% of all patients with TB who have treatment initiated in sub-Saharan Africa die within a year [29], and two-thirds of these deaths may occur in the first 2 months, which reflects the advanced state of illness at the time of final diagnosis. Whereas smear-negative TB has conventionally been regarded as a slowly progressive disease with limited mortality, in HIV-infected cohorts, patients with smear-negative disease often have poorer treatment outcomes and greater mortality than do their counterparts with smear-positive disease [30, 31].

The true magnitude of the problem of smear-negative TB in severely immunocompromised individuals may be underestimated, because many such patients may die before their TB is detected. In Malawi, fully half of the patients with suspected TB in whom a diagnosis was not readily made died while their condition was under investigation [32]. Autopsy studies in South Africa [33], the Ivory Coast [34], Botswana [35], the Democratic Republic of the Congo [36], Kenya [37], and India [38] have revealed TB as the leading cause of death in patients who died with HIV coinfection. Such studies have also shown that the accuracy of predeath diagnosis was poor [39, 40]. Thus, HIV coinfection decreases the sensitivity of microscopy to detect TB at the same time that it increases the need for rapid diagnosis and treatment.

In response to the striking early mortality due to TB in individuals with HIV coinfection and the poor performance of microscopy, health care workers have been driven to use the response to presumptive treatment as a diagnostic method [41]. The specificity of this approach is poor, leading to wasted drug resources, overburdened treatment programs, and mistreatment of many patients with other diseases. Mortality rates among such mistreated patients are high [31].

**DIAGNOSTIC PRIORITIES**

Much is being done to identify HIV-associated TB more quickly by implementing active case finding and abbreviating clinical algorithms; clearly, however, better tests are needed. The greatest need is for tests that can improve the detection of active TB among symptomatic individuals. Better tests could abbreviate the period between the onset of symptoms and the initiation of therapy by being more sensitive, faster to yield results, or simpler to use. An ideal test would combine these features in a simple point-of-care format that could replace microscopy and culture and yield a confirmatory diagnosis at the first clinic visit. Such a test would, of course, have an important impact on both HIV-infected and HIV-uninfected populations.

Improved detection of multidrug resistance (MDR) is also needed. Global surveillance data, when adjusted for prior TB, have not shown a close link between HIV infection and the risk for multidrug-resistant TB (MDR-TB) [42]. However, in some settings in developing countries, striking levels of HIV-related MDR-TB have been found, often related to nosocomial transmission through hospitals and clinics [43, 44]. Recent reports, accompanied by substantial news coverage, of lethal outbreaks of extensively drug-resistant TB (XDR-TB) in South Africa and elsewhere have highlighted the need for rapid methods to identify highly resistant *M. tuberculosis* strains [45]. Lastly, improved detection of latent infection with *M. tuberculosis* in patients with HIV infection, with greater negative and positive predictive values than current tuberculin skin testing, could better target preventive therapy.

**OPPORTUNITIES FOR BETTER DIAGNOSTICS**

A summary of the technical opportunities for detecting HIV-associated TB is presented below, focusing on case detection technologies, drug susceptibility testing (DST) methods, and,
to a limited extent, tools for detecting latent infection (table 1). Methods of DST, which can be performed with a number of case-detection technologies, are described in a separate section.

**Improvements in microscopy.** Microscopic examination of clinical specimens remains the most widely available test for active TB and is the diagnostic centerpiece of the directly observed treatment, short course (DOTS) strategy. Microscopy has the advantages of being inexpensive, relatively rapid to perform, and specific in most settings. Although microscopic detection of acid-fast bacilli (AFB) in sputum is not specific for TB in industrialized countries [46], the vast majority of AFB-positive specimens in TB-endemic countries represent TB, even in settings where nontuberculous mycobacteria may commonly be recovered in culture [8–15, 47].

Although AFB microscopy has changed little over the past 100 years, microscopy in other fields has evolved markedly. Thus far, little has been done to harvest these advances for improved TB diagnostics, and no successful automated microscopy system, for example, has thus far been developed. The 2 common alternatives to conventional direct microscopy that have been adopted in some settings to improve speed or sensitivity are fluorescence microscopy and alternative specimen-processing methodologies.

Fluorescence microscopy, developed three-quarters of a century ago for the detection of TB by exploiting the affinity of fluorochromes (auramine and/or rhodamine) for mycolic acids in the mycobacterial cell wall [48, 49], has been widely used in industrialized countries to improve the speed and sensitivity of AFB microscopy. By use of 25–40× objectives, fluorescence microscopy allows a much larger viewing field than is seen with 100× oil immersion objectives used for carbol fuchsin–stained slides. The sensitivity advantages of fluorescence over light microscopy for the detection of pulmonary TB have recently been confirmed in a systematic review of 45 studies comparing the 2 methods, which found that fluorescence microscopy yielded an average increase in sensitivity of 10%, with no loss of specificity [50]. There are few data evaluating fluorescence microscopy specifically in HIV-infected populations, but there is little reason to believe that the findings would not be similar. Publicly supported field evaluations of various fluorescent microscopy systems are under way to determine performance and feasibility.

Equipment costs limit the wider use of fluorescence microscopes. The microscopes may cost between $10,000 and $20,000 and use an intense ultraviolet light source, such as a high-pressure mercury lamp, which typically costs $100–$300 and lasts only 100–200 h. Light-emitting diodes, which have a life span of >10,000 h and replacement costs that may be less than $5, can also excite dyes such as auramine and rhodamine to fluoresce but have suffered from being considerably dimmer than mercury lamps. Ultrabright light-emitting diodes 30–50 times brighter than standard light-emitting diodes have been developed, and preliminary data have demonstrated the utility of such microscope systems for TB detection [51].

Most microscopic examinations for pulmonary TB are performed directly on smeared and stained preparations of unprocessed sputum, and the search for sputum-processing methods that could improve the yield, safety, or ease of microscopy is many decades old [52]. The poor performance of TB microscopy for individuals with HIV coinfection has given new urgency to this area of work. A variety of chemical and physical methods have been used either to concentrate bacilli by means of gravitational force or filtration or to improve their dispersion in sputum. A recent systematic review of the literature in this area identified 83 studies examining the comparative yield of an alternative method to direct microscopy. The majority of studies that used any procedure for digestion liquefaction followed by centrifugation, prolonged gravity sedimentation, or filtration [53] found an increase in sensitivity of 13%–33% over direct microscopy, when culture was used as the reference standard [54]. Unfortunately, given the variability in patient selection and trial design between published studies, it is not possible to determine which, if any, of these methods is optimal. The easy availability of household bleach (5% sodium hypochlorite), along with its microbicidal activity, make it a commonly studied processing reagent, and multicenter studies to determine the feasibility and impact of wider implementation of a standardized bleach-processing method are planned [55].

**Growth-based detection.** Mycobacterial culture on selective media remains the most sensitive method for detecting *M. tuberculosis* bacilli in clinical specimens and allows subsequent strain characterization, including DST. The slow replication time of *M. tuberculosis* requires that solid media cultures be incubated for 2–8 weeks (depending on the inoculated bacterial concentration) for the growth of the millions of organisms necessary to generate visible colonies. This process can be accelerated by microscopically detecting immature colonies, detecting products of bacterial replication, or using bacteriophages as markers of mycobacterial viability. Several tests have been developed to exploit these principles.

Automated liquid culture systems have been developed to accompany conventional solid media culture. These systems detect bacterial CO$_2$ production or O$_2$ consumption with radiometric, fluorescent, colorimetric, or pressure sensors that allow continuous monitoring, obviate the need for mature colony formation, and roughly halve the time to detection, compared with Löwenstein-Jensen culture [56–66]. Because of the expense of the sophisticated culture vials employed, the usual need for large and expensive incubator/readers, and the recommended requirement for additional backup culture on solid media, these culture systems have seen limited use in TB-en-
Table 1. Tuberculosis (TB) diagnostic technologies, stage of development, and settings of use.

<table>
<thead>
<tr>
<th>Technology, test</th>
<th>Stage of development</th>
<th>Developer(s)/supplier(s)</th>
<th>Level of the health system</th>
<th>DST utility</th>
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<tbody>
<tr>
<td><strong>Case detection</strong></td>
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<tr>
<td>Growth-based detection</td>
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<tr>
<td>Conventional solid media</td>
<td>Commercialized reagents and prepared media</td>
<td>Multiple</td>
<td>Referral</td>
<td>Y</td>
</tr>
<tr>
<td>Automated liquid culture systems</td>
<td>Commercialized, under study for feasibility and impact of use in resource-limited settings</td>
<td>BD, bioMérieux, Trek</td>
<td>Referral</td>
<td>Y</td>
</tr>
<tr>
<td>TK colorimetric media</td>
<td>In evaluation</td>
<td>Salubris</td>
<td>Referral</td>
<td>Y</td>
</tr>
<tr>
<td>MODS assay, thin-layer culture, others</td>
<td>Academic evaluations published</td>
<td>Noncommercial testing methods</td>
<td>Referral</td>
<td>Y</td>
</tr>
<tr>
<td>Phage-based detection</td>
<td>Commercialized, improved test in development</td>
<td>Biotec</td>
<td>Referral</td>
<td>Y</td>
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<tr>
<td><strong>Direct visualization</strong></td>
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<tr>
<td>Conventional microscopy with acid-fast staining</td>
<td>In routine use</td>
<td>Multiple</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td>Fluorescent microscopy with nonspecific cell-wall staining</td>
<td>In routine use</td>
<td>Multiple</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td>Fluorescent microscopy with LED light source</td>
<td>In development</td>
<td>Various</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td>Fluorescent microscopy with molecular probes (FISH)</td>
<td>In development</td>
<td>ID-FISH Technology</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td>Automated microscopy</td>
<td>In development</td>
<td>Various</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td>Computer-assisted microscopy</td>
<td>In development</td>
<td>Various</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td><strong>VOC detection</strong></td>
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<tr>
<td>Electronic nose analysis of headspace gas</td>
<td>In development</td>
<td>Scensive</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td>GC/MS analysis of exhaled air</td>
<td>In development</td>
<td>Menssana Research</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td>Handheld surface acoustic wave-GC</td>
<td>In development</td>
<td>Electronic Sensor Technology</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td>Giant African pouch rats</td>
<td>In evaluation</td>
<td>Apopo</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td>Honeybees</td>
<td>In development</td>
<td>Inscentinel</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td><strong>Antigen detection</strong></td>
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<tr>
<td>TB-derived antigen detection in urine or other clinical material</td>
<td>In development</td>
<td>Chemogen, Proteome Systems, TB DiaDirect, others</td>
<td>Health center</td>
<td>N</td>
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<tr>
<td>TB-derived antigen detection in exhaled air vapor</td>
<td>In evaluation</td>
<td>Rapid Biosensor Systems</td>
<td>Health center</td>
<td>N</td>
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<tr>
<td><strong>Antibody detection</strong></td>
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<tr>
<td>Detection of diagnostic antibody responses to TB</td>
<td>Many commercially available, improved tests in development</td>
<td>Various</td>
<td>Health center</td>
<td>N</td>
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<tr>
<td><strong>Molecular detection</strong></td>
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<tr>
<td>Automated, nonintegrated NAAT</td>
<td>Commercialized</td>
<td>GenProbe, Roche, BD, others</td>
<td>Reference</td>
<td>N</td>
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<tr>
<td>Automated, integrated NAAT</td>
<td>In development</td>
<td>Cepheid</td>
<td>Reference</td>
<td>N</td>
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<tr>
<td>Simplified manual NAAT (LAMP)</td>
<td>In development</td>
<td>Eiken</td>
<td>Microscopy</td>
<td>N</td>
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<tr>
<td>Nonamplified probe detection</td>
<td>In development</td>
<td>Investigen, others</td>
<td>Microscopy</td>
<td>N</td>
</tr>
<tr>
<td>Transrenal DNA detection</td>
<td>In development</td>
<td>Xenomics, others</td>
<td>Referral or microscopy</td>
<td>N</td>
</tr>
<tr>
<td>Manual amplification and hybridization</td>
<td>In evaluation</td>
<td>Innogenetics, Hain</td>
<td>Reference</td>
<td>N</td>
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(continued)
Table 1. (Continued.)

<table>
<thead>
<tr>
<th>Technology, test</th>
<th>Stage of development</th>
<th>Developer(s)/supplier(s)</th>
<th>Level of the health system&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DST utility&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td><strong>Species identification</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Luminescent probe of culture isolate</td>
<td>Commercially available</td>
<td>GenProbe</td>
<td>Reference</td>
<td>N</td>
</tr>
<tr>
<td>Fluorescent probe of smear-positive sputum</td>
<td>In development</td>
<td>ID-FISH</td>
<td>Referral</td>
<td>N</td>
</tr>
<tr>
<td>Reverse hybridization line probe from culture isolates</td>
<td>Commercially available</td>
<td>Innogenetics, Hain</td>
<td>Reference</td>
<td>N</td>
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<tr>
<td>Dipstick detection of TB antigens in positive cultures</td>
<td>In demonstration&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Tauns</td>
<td>Referral</td>
<td>N</td>
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<tr>
<td>Species-specific amplification or sequencing</td>
<td>Research use</td>
<td>Various</td>
<td>Reference</td>
<td>N</td>
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<tr>
<td><strong>LTBI detection</strong></td>
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<tr>
<td>Tuberculin skin test with PPD</td>
<td>Commercialized</td>
<td>Multiple</td>
<td>Microscopy</td>
<td>N</td>
</tr>
<tr>
<td>MPT-64 skin patch</td>
<td>In evaluation</td>
<td>Sequella</td>
<td>Health center</td>
<td>N</td>
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<tr>
<td>Whole-blood IFN-γ release assay</td>
<td>Commercialized; in evaluation for disease-endemic countries</td>
<td>Cellestis</td>
<td>Referral</td>
<td>N</td>
</tr>
<tr>
<td>ELISPOT IFN-γ release assay</td>
<td>Commercialized; in evaluation for disease-endemic countries</td>
<td>Oxford Immunotech</td>
<td>Referral</td>
<td>N</td>
</tr>
<tr>
<td>Skin testing with TB-specific antigens</td>
<td>In early evaluation</td>
<td>Statens Serum Institut</td>
<td>Microscopy</td>
<td>N</td>
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</table>

**NOTE.** DST, drug-susceptibility testing; ELISPOT, enzyme-linked immunospot; FISH, fluorescence in situ hybridization; GC, gas chromatography; IFN, interferon; LAMP, loop-mediated, isothermal amplification; LED, light-emitting diode; LTBI, latent TB infection; MODS, microscopic-observation drug susceptibility assay; MS, mass spectrometry; NAAT, nucleic acid amplification testing; PPD, purified protein derivative; VOC, volatile organic compound.
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| **a** The health care system is divided here for convention into 4 levels: reference laboratory, a national or regional laboratory performing specialty mycobacterial tests, not focused on patient care; referral laboratory, a laboratory with TB-specific expertise performing such tests as TB culture, microscopy laboratory, a laboratory performing only microscopy for TB detection; and health center, a clinical facility not routinely providing any mycobacteriology testing. Listed in the table is the level of intended or appropriate use.
| | | | | |
| **b** Indicates that methodology may also be used to detect drug resistance.
| | | | | |
| **c** Löwenstein-Jensen, Ogawa, 7H10, and other media.
| | | | | |
| **d** Beyond NAATs with species-specific primer sequences.
| | | | | |
| **e** Demonstration is a phase in FIND’s development pathway, coming after evaluation, in which the feasibility and impact of programmatic use are measured.

Demographic countries. The Foundation for Innovative New Diagnostics (FIND) is working in collaboration with the Consortium to Respond Effectively to the AIDS/TB Epidemic (CREATE) and Becton Dickinson to execute large-scale demonstration projects of liquid culture in Zambia, South Africa, and Brazil. These projects will examine the feasibility, impact, and cost-effectiveness of using cost-reduced Mycobacteria Growth Indicator Tube (MGIT; Becton Dickinson) culture for the detection of TB in settings of high HIV infection prevalence.

Potential disadvantages of liquid culture include a high risk of contamination, especially when used by laboratories not experienced in liquid culture of TB, and the lack of colony morphology examination as a protection against unexpected growth of non-TB mycobacteria. A solid media system called “TK,” which is being developed by Salubris in partnership with FIND, uses a proprietary media formulation that allows colorimetric detection of bacterial growth before the appearance of visible colonies [67]. Additionally, contaminating nonmycobacterial species cause a different color shift, to green rather than red, which allows simple discrimination between AFB-positive and contaminated vials. Preliminary studies reported that the TK media system, which is relatively inexpensive to manufacture, detected TB 10 days faster than did conventional Löwenstein-Jensen media in a limited number of cultures [68]. Multicenter evaluations to confirm the performance of TK media have yet to be performed.

Noncommercial methods to speed mycobacterial detection have also been developed. A variety of redox reagents, such as Alamar blue [69], have been used to detect early growth in liquid culture but have not seen widespread use. Similarly, microscopic examination of thin-layer agar plates for the early detection of mycobacterial microcolonies [70] has been proposed, as has microscopic examination of liquid culture. By adding antituberculous antibiotics to adjacent wells and examining for comparative growth, this latter technique (termed the “microscopic-observation drug susceptibility assay”) has
been applied for early detection of drug resistance as well, as is described below in the “DST” section [71].

The ability of mycobacteriophages to infect and replicate in viable M. tuberculosis has been exploited in diagnostic assays. In one, a lytic mycobacteriophage, D29, is used to infect M. tuberculosis bacilli in processed sputum after a short incubation period in media. After the infection period, a virucide is added that kills exogenous phage while not affecting phage internalized within the mycobacteria. Infected cells, once plated on a lawn of the rapidly growing Mycobacterium smegmatis, produce plaques indicating the presence of TB. This biological amplification procedure can be performed in days, compared with weeks for culture, and has theoretically similar sensitivity. This has been developed commercially as the FASTPlaqueTB assay (Biotec Laboratories). In its current form, the test detects 29%–87% of smear-positive disease cases and 13%–78% of smear-negative disease cases within 2 days [72–77]. The capacity of D29 phage to replicate in nontuberculous mycobacteria has not impaired clinical specificity, which remained high (99.1%) even in a study in which 30% of all culture isolates were nontuberculous mycobacteria [74]. Biotec has partnered with FIND to work toward the development of a more sensitive version of the assay. Although the test is laboratory based and involves multiple steps, this assay may offer an alternative to nucleic acid amplification testing (NAAT) for the rapid detection of smear-negative TB at a cost similar to that of culture on commercial media.

The superior performance of culture is accompanied by a range of technical and logistical obstacles that are particularly relevant in the developing world. With the exception of Brazil, the Russian Federation, and South Africa, culture facilities are rare in countries with a high TB burden [78]. Therefore, broadly implementing culture or phage replication as a solution to the problem of smear-negative TB will require significant investment in laboratory infrastructure.

**Antigen detection.** Attempts have been made to detect M. tuberculosis antigens in body fluids, as an alternative to conventional microbiological confirmation. The detection of protein and nonprotein antigens has demonstrated diagnostic utility for a number of other diseases, including malaria, influenza, and bacterial meningitis, but no such test for TB has been successfully commercialized. Theoretical advantages to this approach include quantitative correlation with burden of disease, the likelihood of high specificity, and lack of dependence on a functioning immune response. Assays using serum or urine might have particular application in HIV-associated TB, in which extrapulmonary disease is common and a large total body burden of bacilli may not be reflected in the sputum.

Some attempts at antigen detection for TB diagnosis have used polyclonal antibodies against whole cell or other crude antigen preparations to detect M. tuberculosis antigens in blood, cerebrospinal fluid, or urine [79–82]. More recently, monoclonal antibodies have been developed to target proteins and glycolipids abundant in culture filtrate, such as MPT32 [83], the antigen 85 complex [84–86], the 38 kDa protein [85, 87], and lipoarabinomannan. Lipoarabinomannan is a heat-stable, high-molecular-weight glycolipid present in the lipophilic cell wall of mycobacteria in a variety of forms in and across different mycobacterial species. Several groups have demonstrated measurable concentrations of lipoarabinomannan in the sputum [88, 89], serum [90], and urine [91–95] of patients with TB. FIND is partnering with industry and academic researchers to develop a sensitive and specific commercial lipoarabinomannan detection assay for clinical use.

The only commercial antigen detection test for TB is an assay using the M. tuberculosis–specific antigen MPB-64 (Tauns) as a target for mycobacterial species identification. This simple lateral flow test allows accurate differentiation of M. tuberculosis complex organisms isolated in solid or liquid culture from the remainder of the mycobacterial species. Rapid species identification of culture isolates, most often accomplished using expensive molecular probes, will be important to support the increased use of culture for TB detection in areas where HIV infection is prevalent [96–99]. Unfortunately, the product is currently not available for purchase outside of Japan. FIND is working to make this test available for the public sector in developing countries.

Almost all work on antigen detection has targeted secreted or cell surface antigens present in culture filtrate. Other antigens, expressed in vivo, may also have diagnostic value. Exploratory work at Proteome Systems [100] has identified a number of novel proteins, not found in culture filtrate, that can be detected at low concentrations in body fluids of patients with TB. Additional work in this area may yield reagents targeting an antigen or series of antigens that could be used to develop a point-of-care TB antigen detection test.

**Molecular detection.** Aside from microscopy and culture, the only proven method for detection of M. tuberculosis that has been successfully developed as a clinical diagnostic tool is NAAT. These tests use oligonucleotide primers and enzymes to catalyze reiterated reactions that amplify a target, probe, or signal, yielding a result within minutes to hours. The analytic sensitivity of these systems tends to be exquisite, although clinical performance may vary. Several TB NAAT methods have been developed, and 2 are currently commercialized in the United States. The 3 most widely used of these assays, polymerase chain reaction (PCR; Roche Diagnostics), transcription-mediated amplification (GenProbe), and strand-displacement amplification (Becton Dickinson) have shown excellent specificity and speed, as well as sensitivity approaching but not equaling that of dual-media culture [101, 102]. Despite the clear advantages of NAATs over existing tests, especially for the rapid
detection of smear-negative disease, they have only limited use in TB-endemic settings, primarily because of their cost and complexity. Testing itself is usually performed on expensive, precision instruments that are beyond the capacity of most diagnostic sites to purchase or maintain and that require skilled technologists to operate. Even in established molecular laboratories in resource-limited countries, performance is highly variable, suggesting that much greater assay robustness or much more laboratory support will be needed before NAAT can be implemented more widely [103].

A number of groups have recently investigated methods to simplify sample processing and molecular amplification. Biodefense spending has been an important impetus for this, as has been the market for point-of-care testing in industrialized countries. One exciting technology under development for the detection of TB is the loop-mediated, isothermal amplification (LAMP) method, invented by researchers at Eiken Chemical. The advantages of this technology, which uses 6 specifically designed primers and a single polymerase with strand-displacement activity, are that it requires no thermocycler, is a closed system, and gives a visual readout interpretable by the naked eye [104]. If sufficiently simple to use, such a test might be implemented in microscopy centers to replace or augment microscopy to improve the sensitivity of case detection at this level of the health system. Working with FIND, Eiken is developing a prototype test for TB, using a simplified specimen-processing method that might feasibly be implemented in resource-limited areas. Preliminary data suggest that, in its current form, the assay may be performed at the benchtop by technicians with no molecular training and performs similarly to commercialized instrumented systems, detecting essentially all smear-positive specimens and half of smear-negative specimens [105]. The requirement for specimen processing and DNA extraction is an important obstacle to the implementation of any molecular amplification method in laboratories without substantial technical infrastructure. FIND is working with Cepheid to develop a real-time PCR assay for TB on its GeneXpert platform that automates sputum processing, DNA extraction, gene amplification, and target detection into a single, hands-free test. The assay, which is being developed in collaboration with the University of Medicine and Dentistry of New Jersey, will use molecular beacons to detect the presence of both TB and rifampin resistance in <2 h [106]. The first clinical trials of the test started in May 2007.

The finding that DNA from apoptotic cells in the body make their way into the urine in short fragments (150–200 bp) in predictable and detectable concentrations [107] has led to the development of real-time PCR assays, for pregnancy-related, cancer-related, and transplant-related diseases, that use urine rather than invasively collected material. Testing for transrenal DNA from *M. tuberculosis* has been performed in a preliminary study of 20 patients with TB and TB-negative control subjects. In that study, TB-specific DNA was found in all 20 patients with TB and in none of the control subjects. The results of urine culture for *M. tuberculosis* were negative [108]. The frequency of extrapulmonary disease and paucibacillary pulmonary disease among patients with HIV-associated TB may limit the sensitivity of any sputum-based tests, so urine-based detection of mycobacterial antigens or DNA is highly relevant for this population. Further development of this approach, and fieldwork to evaluate its potential utility in resource-limited areas, is under way.

**Diagnostic humoral immune responses.** Many TB proteins and nonprotein molecules are highly immunogenic. Exploiting the humoral and cellular responses that they generate for a TB diagnostic test is attractive, in part because of the potential simplicity of testing in a dipstick or similar format. Immunologic testing holds the additional theoretical advantage of not depending on access to the infecting bacteria and, thus, detecting extrapulmonary and paucibacillary disease as readily as cavitary pulmonary TB.

Although responses are heterogeneous, antibodies to *M. tuberculosis* antigens can be detected in the majority of immunocompetent patients with active TB. The development of serologic tests for TB based on the detection of such antibodies has been attempted for decades. For years, this work centered on the 38 kDa antigen and a limited number of other antigens abundant in culture filtrate or immunodominant in animals. Although >2 dozen serologic tests based on these reagents are currently being marketed, primarily in developing countries, none of the existing commercial tests shows adequate sensitivity and specificity for recommended use. Given the altered humoral immune responses in patients with HIV-associated immunocompromise, it is not surprising that the performance of these tests is especially poor in HIV-coinfected patients [109–111].

The failure of existing antibody-based TB tests to meet clinical needs does not mean that development of such a tool is not possible. Recent work to look beyond the targets commonly used in current commercial kits has identified a number of promising antigens that show high specificity on initial screening and might be usefully included in a multiantigen assay [112, 113]. More work is needed in 2 areas. First, a systematic discovery effort is needed to identify additional targets among the nearly 4000 proteins encoded by the *M. tuberculosis* genome, particularly those not found in culture filtrate. Protein expression patterns of bacteria vary with the growth conditions of the organism, including in response to immunologic pressure [114], and it is likely that a number of antigens expressed during different stages or types of infection will be needed for a test that is sensitive in diverse clinical settings. For example, despite the general blunting of antibody responses to TB in individuals...
with HIV coinfection [115], some antigens, such as TB9.7 and 81/88 kDa, may be preferentially expressed in individuals with HIV coinfection and have specific diagnostic utility in that population [112, 116]. This points to the second need, which is for greater attention to the breadth and precise nature of disease represented in the collections of clinical samples used to evaluate antigens. Often, antigens are selected or evaluated using serum from populations that are demographically restricted, manifest only specific types of disease (e.g., smear-positive pulmonary TB), or are poorly characterized clinically and microbiologically.

**Diagnostic immune responses.** HIV-related immunocompromise is the most significant risk factor for reactivation TB in individuals with latent TB infection (LTBI). The standard tool, the tuberculin skin test, has a number of limitations, including the need for injection, the subjectivity of readout, and the cross-reactivity of purified protein derivative with bacille Calmette-Guérin and other mycobacteria. Two promising new in vitro tests of cell-mediated immunity use M. tuberculosis-specific antigens encoded by DNA sequences that have been deleted from all bacille Calmette-Guérin vaccine strains and are not present in most species of nontuberculous mycobacteria. QuantiFERON-TB Gold (Cellestis) uses a whole-blood assay to measure interferon (IFN)-γ production from sensitized T cells, and the T SPOT-TB test (Oxford Immunotec) uses an enzyme-linked immunospot assay to quantify the number of peripheral blood mononuclear cells producing IFN-γ in response to antigen stimulation. Both of these assays give objective results, with sensitivity, as measured in patients with active TB, comparable to that of the tuberculin skin test. The lack of cross-reactivity with bacille Calmette-Guérin gives important benefits in specificity, and, in low-incidence settings, both tests give results that more closely associate with the degree of clinical exposure to TB than does the tuberculin skin test. Interesting preliminary data suggest that quantitative responses to ESAT-6 might even be useful in detecting incipient disease in individuals with LTBI [117]. A review of these technologies has recently been published [118].

Whether these IFN-γ assays will show adequate sensitivity in HIV-infected populations to warrant their routine use in endemic settings is an unresolved question, and early field data are promising but conflicting [119, 120]. Although there is also some interest in the use of these tests to rule out TB in symptomatic patients with suspected TB, existing data suggest that, even in largely HIV-uninfected populations, a significant portion of patients with confirmed TB may be test negative [121]. More operational research is needed to clarify the role of these important new diagnostic methods for the control of TB in resource-limited areas, especially where HIV infection is prevalent.

**Sensing volatile organic compounds and other biomarkers.** There is a growing interest in detecting TB by “sniffing” for the presence of characteristic volatile compounds or patterns of compounds in exhaled air or headspace gas over sputum or bacterial cultures. Press reports have highlighted the efforts to use animals, such as the African giant pouch rat (Cricetomys gambianus), to detect TB [122]. Engineered sensing technologies that physically detect volatile organic compounds would be much easier to implement. One such technology is the electronic nose, so called because it mimics a biological olfactory system by using nonspecific gas sensors and pattern recognition algorithms to detect and analyze thousands of different odors with a relatively small number of nonselective receptors [123].

Currently, there are US Food and Drug Administration–approved applications for electronic noses in the detection of urinary tract infection and bacterial vaginosis. Other health applications are under development [124], including for detection of TB, for which there are interesting preliminary data. Fend et al. [125], using a nonoptimized sensor array of 14 conductive polymer sensors, were able to differentiate M. tuberculosis, Mycobacterium avium, and Pseudomonas aeruginosa in headspace gas over spiked sputum. The analytic sensitivity for M. tuberculosis was 10³ cfu/mL. The clinical sensitivity values were 91% and 89% in patient samples that were culture positive (n = 55) or culture negative (n = 79), respectively, for M. tuberculosis. Interestingly, natural and experimental infection with Mycobacterium bovis could also be detected in cattle and badgers by sampling the headspace gas over serum [126].

More quantitative analytic approaches to volatile organic compound detection have been used as well. In a recent study, gas chromatography/mass spectroscopy analysis of exhaled air has been used to distinguish patients with pulmonary TB from healthy subjects and those with other types of lung disease, by using pattern recognition and fuzzy logic [127]. The volatile organic compounds recognized in the breath of patients with TB were structurally similar to those found in headspace gas over TB culture.

**DRUG SUSCEPTIBILITY TESTING**

Drug control efforts in TB-endemic countries have traditionally focused on case detection and not on DST. Ten years ago, the phenomenon of MDR-TB gained attention as high-prevalence foci in developing countries were recognized, prompting the expansion of DOTS treatment strategies to cover MDR-TB (DOTS Plus). Recent recognition of clusters of rapidly fatal cases of XDR-TB has, in a similar way, underscored the need for expanded diagnostic strategies under DOTS.

In most developing countries, DST, where available, is usually performed on solid media, such as Löwenstein-Jensen. Results often come back after 8–18 weeks of waiting, during which time many patients with MDR-TB or XDR-TB may have died, transmitted their disease, or both. There are a number of re-
cently developed methods for the detection of drug resistance that are faster, although usually no less complex, than conventional DST on solid media. Some of these technologies are described below. Unfortunately, the laboratory infrastructure to support such testing in disease-endemic countries is very limited. Of the 22 highest-TB-burden countries, fewer than half have >3 laboratories in the entire national laboratory network with the capacity of performing DST [128]. Thus, efforts to contain drug-resistant TB must focus both on the development of simpler and more rapid detection methods and on the improvement of laboratory capacity.

Growth-based methods of TB detection can usually also be applied to test drug susceptibility, and many of the techniques described above have been successfully adapted for DST [129–135]. Over the past 2 decades, automated liquid culture systems have come into common use in developed countries for DST with both first- and second-line TB drugs, yielding results in 2–4 weeks. Performing DST in these systems directly from smear-positive specimens can further reduce delays in receiving results to 1–3 weeks [56, 136–139]. Noncommercial methods for more rapid DST have also been published, including direct solid media inoculation and readout via colony inspection or by the addition of colorimetric substrates, as in the nitrate reductase assay, or Greiss method [140]. The microscopic-observation drug susceptibility assay, mentioned briefly above, is a noncommercial system based on microscopic observation of early colony formation in liquid media. This inexpensive method yields culture and drug resistance phenotype results in just over a week and has a high correlation with DST using reference methods, especially for rifampin and isoniazid [141]. The feasibility of programmatic implementation of such a method, which is not available in a kit format, has not yet been demonstrated, and its use is currently restricted to research settings.

A version of the phage replication assay that can detect rifampin resistance in 48 h directly from smear-positive sputum has recently been launched commercially (FASTPlaque-Response) and is under evaluation for use in the public sector of resource-limited countries [142]. Genetically engineered luciferase reporter phages have also been developed for rapid DST [143–145]. This latter approach is not currently exploited in a commercial assay and has the disadvantage of requiring a period of culture before testing.

The restricted number of genetic mutations responsible for rifampin resistance has been exploited by 2 currently available commercial molecular assays, GenoType MTBDR (Hain LifeScience) and INNO-LiPA RifTB (Innogenetics), both of which use conventional PCR followed by amplicon hybridization onto a series of oligonucleotide probes on nitrocellulose strips to detect rifampin resistance with a 1-day turnaround. A number of trials with these tests, performed on culture isolates or directly on smear-positive sputum, have shown good correlation with conventional rifampin susceptibility testing [146–153]. The GeneXpert system described above [106] uses similar gene targets but, by automating the processing, amplification, and detection steps, aims to greatly simplify molecular testing, making point-of-care TB detection and broad access to drug resistance screening possible.

**SUMMARY**

In summary, there are a number of exciting technologies being developed for the improved diagnosis of TB, including HIV-associated TB. In the very short term, better case detection can be achieved through revised diagnostic algorithms, improved microscopy, and implementation of rapid mycobacterial culture. In the longer term, completely novel approaches are likely to be available. Both short-term and long-term solutions, if they are to have an impact, will require sustained support of public-private partnerships coupled with a national commitment to improve laboratory infrastructure and rapidly adopt technologies with demonstrated utility.

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