SUPPLEMENT ARTICLE

Tuberculosis Diagnostics and Biomarkers: Needs, Challenges, Recent Advances, and Opportunities

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Tuberculosis is unique among the major infectious diseases in that it lacks accurate rapid point-of-care diagnostic tests. Failure to control the spread of tuberculosis is largely due to our inability to detect and treat all infectious cases of pulmonary tuberculosis in a timely fashion, allowing continued *Mycobacterium tuberculosis* transmission within communities. Currently recommended gold-standard diagnostic tests for tuberculosis are laboratory based, and multiple investigations may be necessary over a period of weeks or months before a diagnosis is made. Several new diagnostic tests have recently become available for detecting active tuberculosis disease, screening for latent *M. tuberculosis* infection, and identifying drug-resistant strains of *M. tuberculosis*. However, progress toward a robust point-of-care test has been limited, and novel biomarker discovery remains challenging. In the absence of effective prevention strategies, high rates of early case detection and subsequent cure are required for global tuberculosis control. Early case detection is dependent on test accuracy, accessibility, cost, and complexity, but also depends on the political will and funder investment to deliver optimal, sustainable care to those worst affected by the tuberculosis and human immunodeficiency virus epidemics. This review highlights unanswered questions, challenges, recent advances, unresolved operational and technical issues, needs, and opportunities related to tuberculosis diagnostics.

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Tuberculosis remains one of the most important causes of death from an infectious disease [1, 2], with the latest World Health Organization (WHO) figures [2] indicating that in 2010 there were 8.8 million incident cases of tuberculosis, with about 13% of tuberculosis cases occurring among people living with human immunodeficiency virus (HIV). There were 1.1 million deaths from tuberculosis among HIV-negative people and an additional 350 000 deaths from HIV-associated tuberculosis. There were 3.2 million incident cases of tuberculosis and 320 000 deaths from tuberculosis among women in 2010. In 2009, there were almost 10 million children who were orphaned as a result of parental deaths caused by tuberculosis. In addition, the global emergence of multidrug-resistant tuberculosis, extensively drug-resistant tuberculosis, and more recently, totally drug-resistant tuberculosis presents a formidable challenge to tuberculosis control, especially in Eastern Europe, Asia, and sub–Saharan Africa [3, 4].

Mycobacterium tuberculosis is transmitted via minute aerosol droplets that remain suspended in the air for prolonged periods of time, posing a particular infection-control challenge. Different outcomes may result following inhalation of an infectious droplet containing *M. tuberculosis* [5]. The probability of development of active clinical tuberculosis after being infected with *M. tuberculosis* is very small. Less than 10% of those infected develop symptoms and signs of active disease over a lifetime; the actual figure depends on geographical location, *M. tuberculosis* strain type, genetic background, immunosuppression, and other risk factors. The majority of immunocompetent individuals either eliminate *M. tuberculosis* or contain it in a latent state in which an equilibrium is established between host and pathogen. Latent *M. tuberculosis* infection is a clinical condition that occurs after an individual is infected with *M. tuberculosis*, the infection is established, and the elicited host immune response contains the *M. tuberculosis* bacilli in a quiescent state, thereby preventing active replication and tissue damage. *M. tuberculosis* bacilli are present in host tissue, yet there are no clinical symptoms or signs of active tuberculosis disease. Importantly, reactivation of latent *M. tuberculosis* bacilli can occur at any time in the infected individual's lifetime, depending on the waning of immunity due to chronic diseases such as diabetes, alcoholic liver disease, HIV coinfection, and use of steroids or other immunosuppressive drugs. Thus, when active disease occurs in later life, it becomes difficult to ascertain whether it is due to reactivation of latent *M. tuberculosis* bacilli or to a new infection with another *M. tuberculosis* strain. Due to the ubiquitous nature of *M. tuberculosis*, WHO estimates that approximately 2 billion people (one-third of the world's population) have been infected with *M. tuberculosis* [6]. Accurate classification of *M. tuberculosis* infection and tuberculosis disease status is essential given that treatment approaches latent *M. tuberculosis* infection and active tuberculosis disease are entirely different.

Currently recommended so called gold-standard diagnostic tests for tuberculosis are laboratory based, and multiple investigations may be necessary over a period of weeks or months before a diagnosis is made [7]. In resource-limited settings, light microscopic examination of Ziehl-Neelsen–stained sputum specimens is often the only tuberculosis test available. It is used mainly for suspected pulmonary tuberculosis cases and is an insensitive technique that performs poorly in young children [8] and individuals who are immunocompromised [7, 9]. It also fails to detect extra pulmonary disease (for which invasive sampling to obtain lymph node aspirate, cerebral spinal fluid, or other specimens may be required) or identify drug resistance [7, 9].

THE NEED FOR MORE ACCURATE AND RAPID DIAGNOSTICS

Despite improvements in tuberculosis control program performance, active tuberculosis case detection rates in many regions remain at unacceptable levels. For example, only 60% of the estimated total tuberculosis caseload is detected in the WHO Africa Region, thus close to half of active tuberculosis cases remain undetected and continue to transmit *M. tuberculosis* [2]. Figure 1 illustrates that in some tuberculosis- and tuberculosis/HIV-endemic countries, less than 4 of 10 cases are detected, with the bulk of HIV-associated tuberculosis cases remaining undiagnosed. Optimal detection of active tuberculosis or latent *M. tuberculosis* infection in HIV-infected individuals remains a major challenge in resource-limited settings. Furthermore, only 7% of the estimated 500 000 new multidrug-resistant tuberculosis patients each year are detected, most of them following prolonged diagnostic delay [3]. Failure to detect drug resistance results in inappropriate treatment and premature death of the individual patient, but it also facilitates amplification of resistance and ongoing transmission within the community, greatly worsening the situation [4, 8]. Although HIV diagnosis has been greatly assisted by the development of robust point-of-care (POC) diagnostics suitable for field use, the diagnosis of tuberculosis remains clinically challenging and logistically difficult in resource-limited settings [11]. A major difference between the 2 diseases is the need to differentiate latent *M. tuberculosis* infection from active tuberculosis disease in tuberculosis suspects, which greatly complicates standard diagnostic approaches. Another difference is their ability to attract commercial investment for product development, as the market for HIV tests is perceived to be considerably more lucrative than that for tuberculosis [12].

In industrialized countries, radiography, other advanced imaging techniques, rapid culture methods, and nucleic acid amplification tests (NAATs) are used to supplement light and light-emitting diode (LED) microscopy for the diagnosis

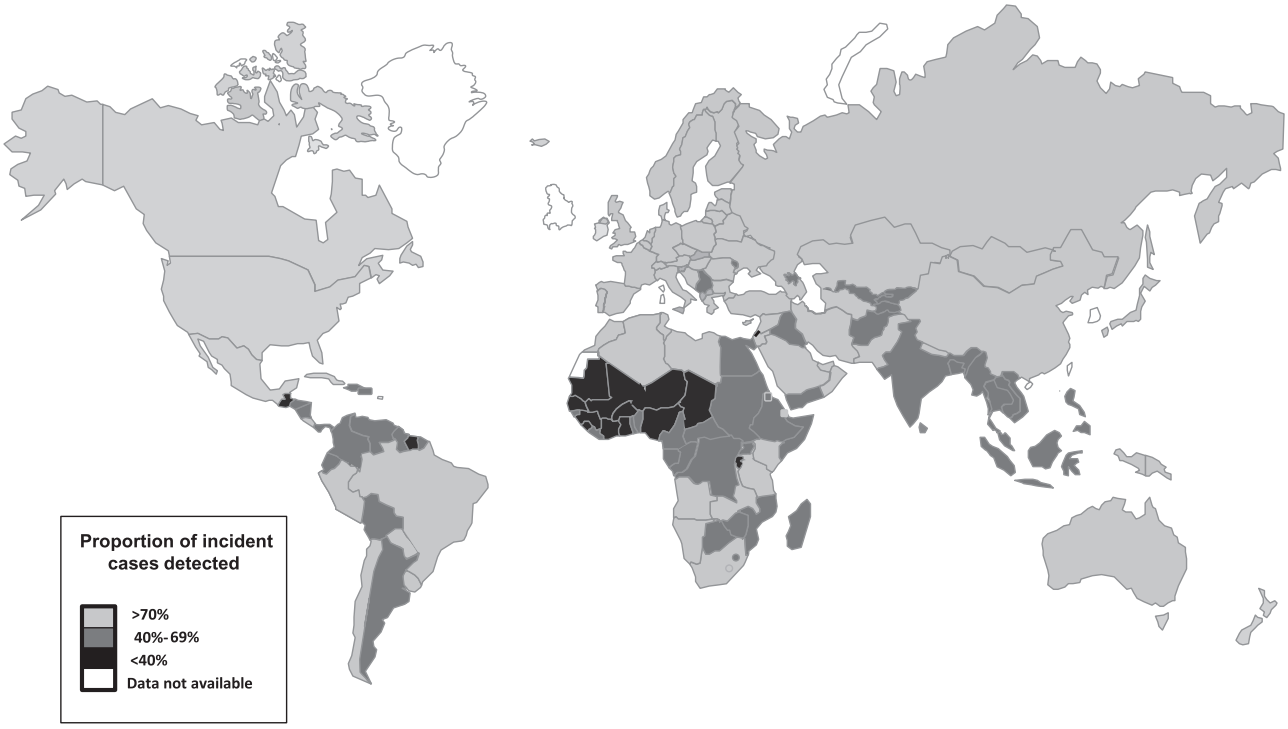

Figure 1. Estimated global tuberculosis case detection rates. Compiled from data presented in the Global Tuberculosis Control Report (WHO. Global tuberculosis control. Geneva: World Health Organization; 2010.).

of active tuberculosis disease [1]. Sensitivity is also enhanced by using tests in combination, induced sputum, or invasive techniques such as bronchoscopy with lavage and tissue biopsies. Unfortunately, many of these technologies are beyond the reach of many of the world's tuberculosis cases. In Africa and Southeast Asia, the WHO regions most heavily affected by tuberculosis, per capita government expenditure on health during 2007 was just $34 and $15, respectively [13]. Although tuberculosis treatment is free, patients are sometimes required to pay for some of the diagnostic tests in the public sector [14, 15], and some patients opt to consult private practitioners at their own cost, which results in multiple visits to confirm a diagnosis, resulting in further expenditures for poorer patients [16, 17]. The limitations of the existing tuberculosis diagnostics toolbox contribute to diagnostic delays with serious consequences for public health efforts to control the epidemic [7, 18]. The vast majority of tuberculosis suspects in endemic countries present to peripheral healthcare facilities that may have no electricity, no running water, and limited or no laboratory facilities. Childhood tuberculosis [9], drug-resistant tuberculosis, and sputum smear–negative pulmonary and extrapulmonary tuberculosis in adults remain the greatest diagnostic challenges [1, 9]. It is estimated that availability of a widely used rapid diagnostic test for tuberculosis that was 100% accurate and that led to initiation of treatment could avert 625 000 tuberculosis deaths each year [18].

IDEAL DIAGNOSTIC TEST CHARACTERISTICS

The ideal tuberculosis test would be a POC device capable of providing an on-the-spot accurate diagnosis of active tuberculosis in HIV-infected and -uninfected adults and children with pulmonary and extrapulmonary tuberculosis; it should also be able to detect resistance to the first-line tuberculosis drugs to avoid initial treatment failure [11] and to allow for rapid prescription of appropriate and specific therapy that prevents the use of inappropriate drugs, thereby inducing drug resistance. The ability of the test to distinguish between active tuberculosis disease and latent *M. tuberculosis* infection is critical. WHO adopted a policy to screen all HIV-infected persons for active tuberculosis as well as latent *M. tuberculosis* infection because tuberculosis remains the leading cause of death among HIV-infected patients and those with latent *M. tuberculosis* infection are at high risk of progression to active tuberculosis in the absence of preventive therapy [19]. The overall impact of any new diagnostic tests for active tuberculosis disease or latent *M. tuberculosis* infection will depend on the extent of their uptake into national tuberculosis programs, affordability both from the patient and health system perspective, the quality and durability of the diagnostic devices, and access to appropriate treatment following diagnosis [20–22]. The need to increase research and development into POC tests for tuberculosis has received

increasing attention in recent years, but there is still a lack of a focused and strategic approach and insufficient integration between areas of biological discovery and test development and the establishment of well-characterized specimen repositories for initial test evaluation [23, 24].

IDENTIFYING ACCURATE AND NOVEL BIOMARKERS

A biomarker can be defined as a characteristic that is objectively measured and assessed as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention [1, 24–27]. Biomarkers can be either host- or pathogen-specific and may advance knowledge by providing information about the pathogenic process, including the current health status and future disease risk of the patient. Thus, there is a need for a specific biomarker to classify patients at a single time point as having active tuberculosis, latent *M. tuberculosis* infection, or no disease [24, 25]. Although these classifications are important from a pragmatic clinical standpoint, there is increasing acknowledgment within the field that these are unlikely to exist as simple compartmentalized states but rather reflect a spectrum [26] that may be profoundly impacted by cofactors that alter the host–pathogen relationship [5]. Other potential applications for biomarkers include predicting future reactivation risk and monitoring the eradication of latent *M. tuberculosis* infection. They may also provide pragmatic endpoints for clinical trials by serving as surrogate markers of cure following tuberculosis treatment or protective efficacy following tuberculosis vaccination. The challenge remains to ensure that any advances made in biomarker discovery translate into diagnostics suitable for implementation in settings that carry the bulk of the global tuberculosis disease burden—in particular, a detection platform that can be implemented as an affordable POC test. Progress in developing these specific tuberculosis biomarkers has been slow [1, 24–28], although several studies are underway using newer technology with multiplexed assays to compare a variety of gene expression profiles among patients with tuberculosis, healthy people with latent *M. tuberculosis* infection, and healthy people with no exposure to *M. tuberculosis*. These studies are measuring several variables with proteomics, transcriptomics, and metabolomics and have been reviewed elsewhere [25, 26]. Defining the correlates of immune protection in individuals with latent *M. tuberculosis* infection presents a major challenge in tuberculosis- and tuberculosis/HIV-endemic areas.

CREATING WELL-CHARACTERIZED SPECIMEN REPOSITORIES FOR NEW TUBERCULOSIS DIAGNOSTICS

Central repositories with well-characterized specimens are critical to identify and validate new diagnostic tests [29, 30]. An independent source of these validation samples is important for several reasons. First, most small companies and academic units will not have sufficient funds to establish a full sample bank, and the lack of a set of validation samples should not be a major barrier to diagnostic test development. Second, the independence of the sample bank will make the validation process more rigorous for the tuberculosis research community. Several banks are currently in existence, but accessibility is highly variable, sample collection processes are not standardized, disease phenotypes are often poorly characterized, and the sample types and volumes are not all clearly defined. This means that developers may arrive at different validation results depending on the sample set used [6]. In addition, samples are rarely collected and stored in a manner suitable for research on metabolic markers or volatile compounds. Addressing such shortfalls will require greater investment that is likely to be beyond the financial and logistical limits of small biotech companies and academic research units. The well-established WHO/Special Programme for Research and Training in Tropical Diseases (TDR) specimen bank, although deficient in important areas, offers the basis of a model that could better meet the needs of the POC tuberculosis test development community [31]. Importantly, it provides an open access resource for developers. However, without substantial investments in strengthening and improving this resource, it will fail to address the needs of the tuberculosis research community. Finally, there is a need to create a pediatric resource, which would be challenging and expensive, but lack of such a resource is a crucial deficiency in current efforts.

IMPROVING SAMPLE COLLECTION AND PROCESSING

The success of any new diagnostic test will depend on the ability to obtain good quality material from the site of disease, which is often not trivial in the environments where POC diagnostics are most needed. Although sputum remains one of the key specimens for tuberculosis diagnosis, the collection of good quality samples adequate for proper diagnosis is difficult, and current sputum processing methods are crude [32]. This represents a particularly important limitation in settings with a high burden of HIV/*M. tuberculosis* coinfection and pediatric tuberculosis because these patients are often unable to produce a quality specimen suitable for analysis [32]. Even if adequate sputum samples are collected, current bacterial decontamination methods probably reduce viable *M. tuberculosis* counts by at least 1 log, whereas mycobacterial concentration in the test sample is suboptimal. Identification of samples other than sputum, such as blood, urine, lung or gastric lavage fluid, biopsies, aspirates or effusions, is critical for improving access to diagnosis for these patient populations. A number of alternative approaches to obtain respiratory

samples exist (ie, nebulizer systems, string test, nasopharyngeal aspiration, lung flute), but these have practical limitations and as a consequence have not been widely adopted in programmatic settings.

RECENT ADVANCES IN TUBERCULOSIS DIAGNOSTICS

Over the past 5 years, several new tests have become available for detecting active tuberculosis disease, screening for latent *M. tuberculosis* infection, and identifying drug-resistant strains of *M. tuberculosis* [1, 25, 27]. The contribution made toward improving case detection and cure rates as well as global control of drug-susceptible and drug-resistant tuberculosis will vary depending on the accuracy, cost, and complexity of the test and on the political will and funder investment available to ensure delivery. Technologies that are not affordable outside of well-funded aid programs or that can only function under referral laboratory conditions are unlikely to reach the mass of undiagnosed tuberculosis in the high-burden countries of Africa and Asia. One approach has been to improve the technologies we already have, making them easier to use. LED microscopes that can be used with fluorescent stains are replacing light microscopy [33]. New approaches are being tested in which patients provide multiple specimens for examination during 1 visit to the clinic, rather than being asked to return at a later date [34]. Front-loading, or so-called 1-day diagnosis, has been endorsed by WHO under defined programmatic conditions.

SEROLOGICAL TESTS

A number of commercial antibody-based tuberculosis diagnostic tests have been developed and are on sale, although clinical validation is usually absent and current test performance is poor [35]. In a comparative study of 19 different tests, the highest sensitivity observed was 59.7%, with some tests detecting less than 1 in 10 tuberculosis patients [35]. A recent meta-analysis commissioned by WHO on currently available commercial serological tests showed very low data quality, inconsistent and imprecise estimates of sensitivity and specificity, and no evidence that existing commercial serological assays improve patient-important outcomes [36]. However, the market for these tests is huge, and they are mainly sold to private practitioners in developing countries where regulatory control of diagnostic tools is lacking.

INTERFERON GAMMA RELEASE ASSAYS

Secretion of interferon gamma by T cells following stimulation with specific *M. tuberculosis* antigens indicates past or current infection with or current disease due to *M. tuberculosis*. Two commercial interferon gamma release assays (IGRAs), the QuantiFERON-TB Gold In-Tube assay (QFT-GIT, Cellestis Ltd, Australia) and the T-SPOT.TB (Oxford Immunotec, UK), which measure interferon gamma released following incubation of patient blood with antigens specific to *M. tuberculosis*, namely early secretory antigenic target-6 (ESAT-6), culture filtrate protein 10 (CFP-10), and the tuberculosis 7.7 antigens, have been developed [1, 25, 36–38]. Despite initial hype following discovery, the ensuing prolific scientific interest, and numerous studies conducted in a variety of clinical situations in adults and children across the world, the 2 IGRAs do not differentiate latent *M. tuberculosis* infection from active tuberculosis disease and are not significantly superior to tuberculin skin tests (TSTs), including in their ability to identify HIV-infected individuals with latent *M. tuberculosis* infection [39]. Recent reviews and meta-analysis concluded that neither IGRAs nor the TST have high accuracy for the prediction of active tuberculosis, although use of IGRAs in certain populations might reduce the number of people being considered for preventive treatment. Thus IGRAs cannot and should not be used in isolation to inform treatment decisions for suspected cases of tuberculosis [40, 41]. UK National Institute for Health and Clinical Excellence guidelines [42] suggest a supportive role for IGRAs alongside traditional TSTs and other routine tuberculosis screening tests in the diagnostic workup of patients suspected of having active tuberculosis. IGRAs are not recommended as tests used to predict active tuberculosis and conduct surveillance of healthcare staff or outbreak investigations, and for contact screening, including screening of children and HIV-infected individuals [40, 41]. WHO advises against the use of IGRAs over TSTs in low- and middle-income countries with typically high tuberculosis and/or HIV burdens [40, 41] as a diagnostic test. An expert scientific panel recently convened by the European Centers for Disease Prevention and Control to clarify these issues and review the scientific evidence base for use of IGRAs in clinical practice concluded that IGRAs should not and cannot replace the existing standard diagnostic methods for the diagnosis of active tuberculosis [40]. The panel also emphasized that a negative IGRA result does not exclude active tuberculosis disease and in high-risk groups, a negative IGRA does not rule out *M. tuberculosis* infection. The expert panel suggested that in order to identify individuals with latent *M. tuberculosis* infection for whom preventive treatment could be considered, IGRAs may be used only in conjunction with an overall risk assessment to provide supplementary information as part of a diagnostic workup.

IGRAs are an improvement over TSTs in that they are less prone to false positives caused by nontuberculosis mycobacteria or bacille Calmette-Guérin have an internal control, and do not require a follow-up visit to assess the reaction. However, IGRAs are often wrongly marketed as tuberculosis diagnostic tests despite their limited clinical utility. They are also expensive and require fastidious sample handling to ensure accuracy. It

has taken over a decade of enormous amounts of effort and time and investment of a large amount of funding and resources into IGRA evaluation studies across the globe to reach these recommendations, which will not have a major impact on achieving tuberculosis control. The results also do not reflect the initial excitement, hype, and expectations after IGRAs were launched as a major breakthrough in tuberculosis diagnostics. This also exposes the desperate state of appropriate evaluation of tuberculosis diagnostics in well-designed research studies conducted with the required scientific rigor. It is vital, therefore, that evaluation of any new diagnostic tests does not suffer from a protracted and costly, yet scientifically invalid, evaluation process. There is a dire need to have comprehensive networks of funders, multidisciplinary researchers, and good clinical/good laboratory practice–capable field sites in several geographical locations that could undertake such evaluations using standardized protocols. Industry involvement in diagnostic test evaluation is another primary concern and a potential confounder of results and reason for publication bias.

FLOW CYTOMETRY ASSAYS OF THE *M. tuberculosis*–SPECIFIC T-CELL RESPONSES

Polychromatic flow cytometry has been used to define the functional profile of *M. tuberculosis*–specific T-cell responses in cohorts of patients with active disease and latent *M. tuberculosis* infection [43]. The panel used included a viability marker, CD3, CD4, and CD8 to determine T-cell lineage, and interleukin 2, tumor necrosis factor alpha (TNF-α), and interferon gamma antibodies to comprehensively assess the cytokine functional profile. The proportion of single TNF-α *M. tuberculosis*–specific CD4 T cells were found to be the strongest predictor measure of discrimination between active disease and latent *M. tuberculosis* infection. The sensitivity of the CD4 T-cell signature was 100% and the specificity was 96% for the data generated from a European cohort [43]. Other flow-cytometric-based assays may include major histocompatibility complex (MHC) class I and MHC class II tetramer complexes that allow direct visualization, without ex vivo manipulation, of $CD8^+$ and $CD4^+$ T-cell responses directed against defined MHC–*M. tuberculosis* peptide complexes [44–46]. T-cell responses can now be visualized without the need to show T-cell function.

NUCLEIC ACID AMPLIFICATION TESTS

The most significant advance toward a POC test for tuberculosis has come in the field of nucleic acid amplification with the launch of the GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA) [47, 48]. The GeneXpert multifunctional diagnostic platform is an automated closed system that performs real-time polymerase chain reaction (PCR), producing results in less than 2 hours. The assay is capable of detecting the *M. tuberculosis* complex while simultaneously detecting rifampicin (RIF) resistance (targeting the RIF resistance–determining region of the *rpoB* gene and associated *M. tuberculosis*–specific flanking regions). Disposable cassettes are used, and following a manual sample liquefaction step, the test is fully automated. The test is easy to use and does not require specialist training other than very basic use of a computer. Assay sensitivity is higher than that of smear microscopy and close to that of culture [47–54]. When testing a single sputum sample, the assay detects 98%–100% of sputum smear–positive disease and 57%–83% of smear-negative disease among prospectively studied tuberculosis suspects [52]. In addition, the assay has utility in diagnosing extrapulmonary tuberculosis from a range of samples from extrapulmonary sites, with sensitivities of 53%–95% [55]. The assay is also highly specific with no cross-reaction with nontuberculous mycobacteria or normal flora of the respiratory tract [50]. Among children with culture-confirmed pulmonary tuberculosis, the assay rapidly detected all smear-positive cases and 61% of smear-negative cases when testing 2 induced sputum samples [56]. The assay has also been assessed for screening patients prior to antiretroviral therapy in South Africa. Case detection increased by 45%—from 28% using smear microscopy to 73% using Xpert MTB/RIF assay [57]. Routine screening of all patients prior to antiretroviral therapy is likely to be a highly cost-effective strategy in this very high-burden setting.

Despite the findings of very high sensitivity and specificity for detection of RIF resistance in the initial multicountry evaluation [47], several studies have since detected numbers of false-positive cases of RIF resistance when compared with other testing methods and to *rpoB* gene sequencing [53, 57] in settings with low RIF resistance prevalence. The current WHO implementation guide recommends use of a second testing method to confirm RIF resistance in these settings and to test for resistance to second-line drugs whenever multidrug resistance is detected [58].

Thus, while the Xpert MTB/RIF assay represents a very sensitive diagnostic test for RIF resistance in high-risk populations groups as well as a very sensitive screening test for RIF resistance in low-risk populations, it cannot universally be applied as a definitive test for point of care [59–62].

The Xpert MTB/RIF assay was shown not to be associated with generation of infectious bioaerosols and resulted in a lower biohazard risk compared with that of conventional smear microscopy [49]. This suggests that the assay might reasonably be done without the need for special biosafety equipment, which is lacking in most resource-limited settings. A subsequent multicountry implementation study found that the assay could be successfully implemented at the district and subdistrict level in urban settings, greatly accelerating diagnosis and start of tuberculosis treatment and reducing the

proportion of untreated disease [52]. The Xpert MTB/RIF assay was rapidly endorsed by WHO in December 2010 for use in tuberculosis-, multidrug-resistant tuberculosis-, and tuberculosis/HIV-endemic regions using a risk-based approach to testing [58].

The new test has generated much excitement in tuberculosis diagnostic circles, although some operational concerns remain. [59–62]. Although this is a long-awaited breakthrough for tuberculosis diagnostics, the rate-limiting steps of this new technology are that this device may not be useful where infrastructure limits operation and maintenance of the real-time PCR platforms and associated personal computer. Furthermore, required annual instrument maintenance may be another hindrance. Various corrective measures have been introduced, including revisions to the diagnostic platform software and redesign of one of the cartridge oligonucleotide probes. As part of the routine product improvement cycle, minor changes were made to the Xpert MTB/RIF assay during the course of the last 12 months. The new software and cartridge combination, called G4 version cartridge, has just been released [63]. Although the Xpert MTB/RIF assay is a major advance of a new generation of easy-to-use nucleic acid amplification tests, other amplification technologies are required that do not require thermocycling and have potential to improve on the Xpert MTB/RIF assay, including detecting resistance to drugs other than RIF.

DIAGNOSTIC TESTS FOR ACTIVE TUBERCULOSIS IN CHILDREN

In endemic areas, an estimated 15%–20% of tuberculosis cases are children [2]. Although older children (>10 years of age) often develop adult-type disease and should access sputum-based diagnostic services, young children who are unable to expectorate carry the greatest disease burden [8]. In addition, young children rarely develop the lung cavities typical of adult tuberculosis, resulting in reduced organism loads compared with adult patients. Collecting respiratory specimens from young children is challenging. Gastric aspirates and induced sputum are frequently used in combination to optimize the yield [9, 64, 65] but may require hospitalization. Creative strategies for collecting effective respiratory specimens linked to maximal extraction techniques and sensitive analysis tools are urgently needed. Refining the string test may assist specimen collection [66], whereas microparticle filters and magnetic beads may offer improved organism concentration [67] compared with standard centrifugation but have not been tested in pediatric specimens.

Initial studies using the Xpert MTB/RIF assay excluded children [19], but subsequent studies have shown substantial utility for rapid diagnosis of culture-confirmed cases [56]. Most of the clinically suspected pediatric tuberculosis cases are not confirmed by culture [53], and identifying the optimal reference standard against which novel diagnostic tests should be measured poses a major dilemma. Clear, well-defined case definitions with high sensitivity and specificity are required for conducting evaluation studies of new diagnostics in children [68]. Where the presence of *M. tuberculosis* is not confirmed, the value of a positive new test when the conventional gold-standard test is positive will require further study. Autopsy and biopsy studies may provide invaluable insight into evaluation of new diagnostic tests under these circumstances and resolve the difficulty of distinguishing between a false positive or true positive interpretation for determining specificity and sensitivity [69]. The Xpert MTB/RIF assay has been shown to be applicable to nonsputum samples from patients with extrapulmonary tuberculosis [70] and thus may yield improved diagnostic rates in children. Nonrespiratory specimens such as cerebrospinal fluid are also paucibacillary, and diagnostic yield may be improved with adequate preconcentration of these samples [70]. Some advances have been made, such as recognition that fine-needle aspiration biopsy offers a minimally invasive technique with excellent mycobacterial yield and the ability to rule out important alternative diagnoses in children with a peripheral lymph node mass [71, 72]. This can be done in a decentralized fashion using rechargeable battery–operated LED fluorescent microscopy [73]. For children with uncertain disseminated (miliary) tuberculosis, bone marrow biopsy may assist histological or microbiological confirmation [74]. If adequate specimens are collected, both commercial and noncommercial liquid culture techniques, such as microscopic observation for drug susceptibility testing (MODS), may assist to optimize the yield [75].

Due to the wide spectrum of intra- and extrathoracic disease in children and the nonspecific nature of most signs and symptoms, diagnostic algorithms perform poorly. However, careful exposure assessment, together with accurate symptom characterization and standard chest radiography, provides a diagnosis in the vast majority of children [76, 77]. Tuberculosis meningitis is the most common form of childhood meningitis seen in some tuberculosis-endemic areas where *Haemophilus influenzae* type B and conjugated pneumococcal vaccines are provided. Diagnosis is frequently missed in resource-limited settings. A consensus research case definition was published recently and should be applicable irrespective of the patient's age and HIV infection status or the resources available in the diagnostics research setting [78].

URINE-BASED DIAGNOSTIC TESTS

Urine represents a clinical sample that is easy to collect from both adults and children and has been used extensively to evaluate several antigen and DNA detection assays [79, 80]. Commercially available assays are able to detect lipoarabinomannan (LAM) in the urine of patients with tuberculosis.

Although the sensitivity of this test has been disappointing in non-HIV–infected patients, moderate sensitivity and high specificity has been observed in HIV-infected patients with advanced immunodeficiency [81]. This assay could provide collateral evidence of tuberculosis in patients with advanced HIV infection being screened in antiretroviral therapy clinics and in those with possible disseminated disease. Although the sensitivity of many tuberculosis diagnostic tests declines steeply in HIV-infected patients with more advanced immunodeficiency, paradoxically, the sensitivity of the LAM enzyme-linked immunosorbent assay (ELISA) increases at lower CD4 lymphocyte cell counts [82, 83]. More than two-thirds of tuberculosis patients with CD4 cell counts <50 cells/μL have LAM antigenuria that is rapidly detectable. A cheap POC lateral flow (Determine TB-LAM Ag urine dip-stick test) has now been developed, which provides a qualitative (yes/no) readout of a tuberculosis diagnosis. This assay represents a significant advance, permitting a rapid, low-cost ($3.50 per test), and specific diagnosis of tuberculosis to be made at the point of care in patients with very advanced HIV-associated immunodeficiency. In a study evaluating this test as a tool for tuberculosis screening among patients enrolling in an antiretroviral therapy clinic in South Africa, the sensitivity was equivalent to that of the ELISA format of the assay, and specificity was ≥98% overall and in all patient subgroups stratified by CD4 cell count [84]. Although its use appears to be limited to those with advanced HIV infection, these are the very patients for whom the need for rapid diagnosis is greatest.

TESTS FOR DRUG-RESISTANCE SCREENING

Drug-resistant (single drug-, multidrug-, extensively drug-, and totally drug-resistant) tuberculosis is now well established throughout the world [1–3, 8]. Phenotypic (culture-based) and genotypic (nucleic acid amplification testing–based) methods have been developed to detect drug-resistant tuberculosis, but first-generation tests were rarely available in tuberculosis-endemic areas, were poorly standardized, and had slow turnaround times. Recent advances have changed this situation [85–89]. Genotypic drug-susceptibility testing (DST) for first-line agents is accurate for RIF and isoniazid (INH) but less reliable for streptomycin, ethambutol, and pyrazinamide. One of the most important drugs in the short-course treatment of tuberculosis is RIF, and RIF resistance is a reliable indicator of multidrug-resistant tuberculosis because RIF resistance in the absence of INH resistance is rare, at least in settings using high-quality, fixed-dose combination tablets for treatment. Accurate genotypic DST for other first- and second-line tuberculosis agents remains technically challenging, but improved multiplex-PCR and improved multianalyte detection technology should make genotypic DST a more powerful technique in the future.

Automated liquid culture systems and molecular line probe assays are recommended by WHO as the current gold standard for first-line DSTs [88, 89]. Commercial automated liquid culture DST methods are highly accurate but are expensive, require special equipment and laboratory infrastructure, and remain slow. The most commonly used commercially available automated liquid culture DST system is the BACTEC MGIT 960 system with the BACTEC MGIT 960 SIRE kit (Becton Dickinson, Franklin Lakes, NJ). Microscopic observation for drug susceptibility [87] is a noncommercial test that utilizes the markedly faster growth and microscopic cording appearance of *M. tuberculosis* in liquid media to diagnose *M. tuberculosis* bacilli and provide simultaneous INH and RIF susceptibility testing. Tests such as MODS, the nitrate reductase assay, and colorimetric reductase methods have been conditionally approved by WHO for use at national tuberculosis reference laboratory level [88]. Second-line DST testing is complex and expensive and thus not available in most high tuberculosis–endemic countries. Liquid culture DSTs for fluoroquinolones and injectables have relatively good reliability and reproducibility; however, DSTs for other second-line drugs (ethionamide, prothionamide, cycloserine, terizidone, para-aminosalicylic acid, clofazimine, amoxicillin-clavulanate, clarithromycin, linezolid) are not recommended. Unfortunately, tuberculosis patients in most high tuberculosis–burden countries are rarely screened for drug resistance due to lack of laboratory resources, high cost, and failure to appreciate its relevance.

CHALLENGES OF DEVELOPMENT, EVALUATION, AND IMPLEMENTATION OF NEW TUBERCULOSIS DIAGNOSTIC TESTS

One of the main barriers to POC test development for tuberculosis has been the historical lack of interest, with funding directed largely to new drug or vaccine initiatives and other diseases such as HIV [90]. Examination of research publications listed by the publications database search engine PubMed during the last 5 years (2006–2011) suggests that less than 2% of the published articles on tuberculosis diagnostic research were focused on POC tests (Figure 2). With the advent of the Xpert MTB/RIF assay, a proliferation of publications of the evaluation of the assay at points of care in various clinical groups and geographical settings is anticipated. It is important that lessons are learned from the experience of the evaluation of IGRAs over the past decade, and properly designed and executed trials are conducted. Further basic science breakthroughs are required to develop novel POC technology, and the need for simpler tests that will improve access to diagnosis and care in tuberculosis-endemic areas requires constant emphasis and increased financial investment.

The complex nature of *M. tuberculosis* infection and its interaction with the host remains poorly understood. Recent

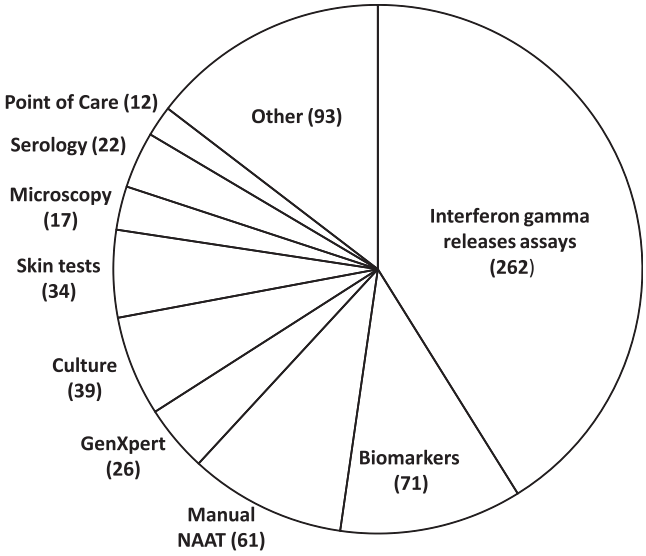

Figure 2. Results of PubMed (US National Library of Medicine) was searched using the terms "tuberculosis" and "diagnosis" and "test" for articles published between 1 January 2008 and 31 December 2011. After exclusion of inappropriate articles, duplications and reviews the articles were reviewed and classified according to the diagnostic topic(s) or technology(ies) they address. Point of care tests were defined as a rapid test providing immediate results without referral to a laboratory or specialist facility. The automated GenXpert assay is classed separately to the manual nucleic acid amplification technologies (NAAT).

studies have found considerable variation in the antibodies expressed by tuberculosis patients, suggesting that multiple targets will be needed if antibody-based tests are to achieve the required sensitivity [91, 92]. The diagnostic potential of secreted antigens and alternative biomarkers such as metabolites also need to be explored. Once suitable markers have been identified and validated, detection platforms must be developed that are easy to use, safe, robust and affordable. Technological challenges include the need for minimal maintenance and operator dependence, as well as the ability to withstand highly variable ambient temperatures, humidity, and dust. There are also logistical difficulties to overcome relating to safety and working with a highly infectious pathogen. Variables that may affect the tests' overall performance and health impact include HIV prevalence, *M. tuberculosis* strain diversity, different environmental and genetic factors affecting particular communities, prevalence of specific drug resistance–conferring mutations, patient-related diagnostic delays, and health system factors such as treatment provision and default rates. One of the most pressing problems in tuberculosis diagnostics is the lack of scientific rigor in manufacturer-driven validation of new tests, compounded by the failure of regulatory bodies to adequately assess test accuracy and appropriate implementation strategies, which allows substandard technologies to be marketed.

OPTIMISM AND FUTURE OPPORTUNITIES

Despite many challenges, prospects for an ideal POC tuberculosis test are improving. With increasing recognition that better tools are essential for early diagnosis and improved disease control, there is mounting pressure on funding bodies to invest in biomarker discovery and diagnostic research. Current efforts focus on hand-held molecular devices, breath- and urine-based assays for the detection of volatile organic compounds, microchip technologies, and proteomics- and metabolomics-based approaches for development of accurate tests, both for diagnosis of tuberculosis disease and latent *M. tuberculosis* infection [1, 24–27, 93]. It has also been acknowledged that traditional market-led manufacturing failed to provide the tools needed to control diseases of global importance that predominantly affect poor people such as tuberculosis, and that future development of tuberculosis diagnostics will benefit from innovative public–private partnerships [11]. In 2008, the World Health Assembly adopted a Global Strategy and Plan of Action on Public Health, Innovation and Intellectual Property, which has the aim of increasing product development in developing countries.

DILEMMAS AND PRIORITIES FOR THE FUTURE

The need for cheap, accurate, rapid, sustainable POC tuberculosis tests has never been greater because early diagnosis is the key to breaking the transmission cycle that sustains the tuberculosis epidemic [11]. There is a general consensus in the Stop TB Partnership movement that to conquer tuberculosis we need to detect early pulmonary disease and provide appropriate treatment. It is also recognized that to increase access to diagnosis for the most vulnerable populations, improved diagnostic tools that can diagnose at points of care without referral to a laboratory or skilled technical personnel are needed. There is less agreement among experts, however, on how to make this happen. In the past 5 years, WHO has endorsed several technologies for tuberculosis diagnosis. The majority of these have been molecular- or culture-based technologies that require considerable laboratory infrastructure. To implement these technologies, a program of laboratory strengthening is being pursued in selected tuberculosis-endemic countries. The resultant centralizing of services presents a dilemma for health planners: should they invest in a flagship laboratory with the latest equipment or should they prioritize peripheral laboratories where the majority of the population seeks care? Alternatively, this may not be a question of replacing conventional laboratory capacity but rather a question of positioning diagnostics at the best-suited level [94]. It must be emphasized that all countries still need conventional culture and DST capacity. One of the immediate priorities is rapid policy reform at the country level to ensure optimal uptake of new diagnostics and the

acceptance that one size no longer fits all. Implementation of new tools requires careful assessment at the country level of underlying epidemiology, existing resources, and cost effectiveness of different diagnostic approaches [93].

CONCLUSIONS

With limited finances, priority must be given to the development of technologies that will reach those not being served by current diagnostic provision. It is crucial to understand that the development of any new, cheap, and more sensitive POC diagnostic tests that have been proven in scientific studies and are applicable at points of care and could facilitate progress toward tuberculosis control will require political commitment and resources for introduction and implementation into high-quality, sustainable, national tuberculosis programs. Meanwhile, more emphasis and attention is required for optimal usage of currently available diagnostics to improve active tuberculosis case detection rates. Simply increasing case detection rates through existing diagnostics will go a long way in reducing tuberculosis transmission.

Notes

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