Diagnostic Tests for Childhood Tuberculosis

Past Imperfect, Present Tense and Future Perfect?

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Over the past decade, there has been significant progress in developing new diagnostic tools for childhood tuberculosis (TB). However, there are still many unan-

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The true global incidence and prevalence of childhood TB remain uncertain, largely because microbiological confirmation of active TB (also called TB disease) is not obtained in the majority of children for a number of reasons. First, children typically have paucibacillary disease, which hampers detection of Mycobacterium tuberculosis. Second, respiratory samples are difficult to obtain in young children and few healthcare facilities are set up to obtain induced sputum samples in routine practice, which have been shown to increase detection yields. Consequently, worldwide the majority of children with active TB are started on anti-mycobacterial treatment based on history, symptoms and clinical signs—with or without supporting radiological findings—alone. Until recently, the World Health Organization (WHO) and other public health agencies made little effort to capture children with non-microbiologically confirmed active TB, as official figures for TB incidence were simply based on the number of smear- and culture-confirmed cases. The WHO Global Tuberculosis Report in 2012 was the first to include estimates for childhood TB. The latest edition (2014) includes estimates for new TB cases in children (an estimated 550,000 cases) and TB-related deaths in HIV-negative children (an estimated 80,000 cases). Notably, the report does not include an estimate for TB-related deaths in HIV-positive children, which is likely to eclipse the figure in HIV-negative children. However, the WHO estimates are based on detection rates in adults, and therefore likely considerably underestimate the burden of TB in children. It is therefore not surprising that a recent study based on mathematical modeling arrived at substantially higher figures, estimating the annual global incidence of active TB in children to be greater than 650,000 cases.

ACTIVE TUBERCULOSIS VERSUS LATENT TUBERCULOSIS INFECTION

Traditionally, infections with M. tuberculosis have been categorized into active TB and latent TB infection (LTBI). Patients with active TB typically have symptoms and/or signs, which depend on the site of the infection, and the infection may be confirmed by conventional (ie, microscopy or culture) or molecular (eg, PCR) microbiological methods (depending on the infection site, adequacy of the sample and the methods used). In contrast, classical dogma suggests that patients with LTBI are asymptomatic, and that their immune system is
containing *M. tuberculosis*. Consequently, current microbiological methods are unable to detect the mycobacteria, and the diagnosis of LTBI is therefore solely based on immune-based tests that detect memory T cells (and potentially other immune cells) induced by exposure to antigens expressed by *M. tuberculosis* [ie, either tuberculin skin test (TST) or interferon-gamma release assay (IGRA)]. The segregation between active TB and LTBI remains useful from a clinical as well as a programmatic perspective as this distinction currently determines the treatment approach [ie, treatment with 1 or 2 antimycobacterial drugs for LTBI vs. 3 or more drugs for active TB]. However, there is increasing evidence that active TB and LTBI are not discrete infection states, but rather opposite ends of a continuum. Historical data from before the advent of antimycobacterial drugs illustrate that some patients with active TB survive without treatment; more recent data show that these asymptomatic survivors maintain TB-specific immunological memory for decades.9 Thus, if tested with immune-based tests (TST or IGRA) after recovery, such individuals would currently have to be classified as “LTBI.”9 Furthermore, recent studies in both nonhuman primates and humans using newer radiological methods, such as positron emission tomography, show that disease activity can be detected in a significant proportion of individuals with a positive immune-based test who are asymptomatic, and therefore would be classified as “LTBI” according to current criteria.10 It is uncertain whether those individuals would convert to overt “active TB” if left untreated. This question will likely remain unanswered given that withholding anti-mycobacterial treatment under those circumstances would be unethical. In addition, several recent publications have described patients with “subclinical TB disease,” who are clinically asymptomatic, but have positive sputum cultures (with or without smear-positivity).10,11 Despite these issues, for clarity we will continue to use the terms “active TB” and “LTBI” in this review.

**IMMUNE-BASED TESTS AND THEIR LIMITATIONS**

**Tuberculin Skin Test**

Since the early 20th century, purified protein derivative (PPD), a heterogeneous mixture of mycobacterial peptides, has been used as the test substance for the TST (also called Mantoux test). The TST is commonly used to support the presumptive diagnosis of active TB, and was the only available test for the detection of LTBI, until IGRA became commercially available in 2002. The key limitation of the TST lies in its limited specificity. False-positive results can occur as a result of prior BCG immunization or infection with nontuberculous mycobacteria (NTM), as both BCG and NTM express peptides that are present in PPD. False-negative results can also occur as a result of immunodeficiency, immunosuppression, malnutrition and errors in test administration or reading. The test requires reading after 48 to 72 hours, which is inconvenient for both healthcare providers and patients; if reading is not performed within this time window, the validity of the test result becomes questionable. Despite those limitations, the latest recommendations of the American Academy of Pediatrics Committee on Infectious Diseases state that for LTBI screening in children less than 5 years of age TST should be used in preference of IGRA.12 However, the recommendations also highlight that the combined use of TST and IGRA results in an increase in diagnostic sensitivity.

An additional major limitation of the TST remains the subjectivity in the reading of the resulting induration, the extent of which determines the test result. The current standard technique for reading—the “ball-point technique”—involves palpating for the outer edges of the induration, marking these with a ballpoint pen and measuring the diameter of the induration using a flexible ruler or a caliper. This technique is prone to considerable inaccuracy with both intra- and interobserver variability, particularly when the induration is not circular.13 Optimization and standardization of the reading technique to achieve greater accuracy and reproducibility have recently been investigated using a variety of novel approaches, including measurement by Doppler imaging, spectrophotometry and ultrasound.14

Another significant problem with the TST are recurrent shortages of PPD. Worldwide, only a small number of manufacturers produce PPD for clinical use, so supply shortages at 1 manufacturer can affect the global market. Following production problems at Evans Vaccines in 2003, the UK had to source PPD from the Statens Serum Institut (SSI) in Denmark as an “unlicensed medicine.”15 In 2013, there was a national shortage of PPD in the US caused by production problems at Sanofi Pasteur (Tubersol) leaving only 1 FDA-licensed product (Aplisol; JHP Pharmaceuticals).16 Later that year, 29 of 52 US jurisdictions reported a shortage of at least 1 of the 2 PPD products “to the extent that routine activities were being threatened or had been curtailed.”17 Canada also experienced a less well-publicized PPD shortage in late 2012.18 A further, Europe-wide shortage of PPD was highlighted by a recent survey of TB experts based in 23 different European countries.19 Sixty percent of these (from 14 different countries) reported a PPD shortage at the time the survey was conducted (June to July 2014). The majority of those reporting a PPD shortage were using RT23 (SSI; 81.0%); fewer reported shortages of Tubertest (Sanofi Pasteur; 9.5%); PPD Tuberculin (BullBio; 4.8%), and PPD Tuberculin (St. Petersburg Institute of Vaccines and Sera; 4.8%).

**Interferon-gamma Release Assays**

Currently, 2 commercial IGRA are available for clinical use, the QuantiferON-TB Gold In-Tube (QFT-GIT, Cellestis; Qiagen, Carnegie, Australia) and the T-Spot.TB assay (Oxford Immunotec, Abingdon, UK). Although IGRA are solely cleared for the diagnosis of LTBI, in clinical practice they are commonly used to support a presumptive diagnosis of active TB. Both assays rely on the detection of interferon-gamma secreted by memory T cells following stimulation with mycobacterial antigens. Both assays incorporate the relatively *M. tuberculosis*-specific RD1 peptides antigens early secretory antigenic target 6 (ESAT-6) and 10kDa culture filtrate protein (CFP-10); the QFT-GIT incorporates and additional antigen, TB7.7. Based on the test principle, IGRA’s likely have greater specificity than the TST and are not confounded by prior BCG immunization, as all 3 stimulatory antigens are absent from all BCG vaccine strains in current use. Also, the impact of current or previous NTM infection and/or exposure on test specificity is relatively limited, since only a small number of NTM express ESAT-6 and CFP-10.20 However, (“false”) positive IGRA results have repeatedly been reported in individuals infected with *Mycobacterium kansasi*, *Mycobacterium marinum* and *Mycobacterium szulgai*.21,22

Although initially heralded as a tool that has the potential to revolutionize the diagnosis of TB, there are now legions of publications highlighting the limitations of IGRA.12,23-25 First, similar to the TST, the performance of IGRA in patients with immunodeficiencies or receiving immunosuppressive treatment is overall poor (the extent of which depends on the type of the immunodeficiency and the degree of immunosuppression), and false-negative assay results remain a significant problem in these patient groups.26,27 This problem is further compounded by those patients also being at greatest risk of progression from LTBI to active TB, making the accurate identification of latent infection in those individuals crucially important.22,25 As there is no gold standard for the diagnosis of LTBI (with the TST having previously been regarded as the gold standard) the true sensitivity of IGRA for the detection of LTBI cannot be determined. Nevertheless, several robust meta-analyses on the performance of IGRA as a supportive tool for the diagnosis...
of active TB have shown that IGRA perform no better than the TST in this setting, reporting a pooled sensitivity of approximately 60–80% in immunocompetent individuals, and even lower pooled estimates in HIV-infected patients.12,20

There is now a considerable body of evidence that the performance of IGRA is worse in young children compared with adults.12 Although the precise underlying mechanisms remain uncertain, it is likely that incomplete immune maturation plays a significant role.34 Indeterminate IGRA results (resulting from failed negative or positive control samples), which convey no information regarding the TB infection status of the patient, are significantly more common in young children than in adults.32–34 One pediatric study reported that indeterminate QFT-GIT results occurred in 83 (35%) of the 237 study participants.26 However, in that study, a large number of children were immunodeficient or were receiving immunosuppressive medication. The majority of pediatric studies in countries with low HIV prevalence have reported lower rates of indeterminate IGRA results, generally ranging between 5% and 20%.2,3,32–34 In addition to young age and immunodeficiency/immunosuppression, further factors that have been found to be associated with indeterminate IGRA results include malnutrition, chronic renal disease, autoimmune conditions, malaria and co-existing helminth infections.32,33 Preanalytical sources of assay variability that can result in indeterminate assay results include delays in sample incubation and inadequate shaking/mixing of QFT-GIT tubes.32,38

Further limitations of IGRA include their relatively high cost and the need for adequate laboratory facilities, which precludes their use in many resource-limited, high TB prevalence settings. In addition, several studies have shown that the reproducibility of IGRA is suboptimal when serial testing is performed (ie, with unexplained conversions from positive to negative and vice versa).12 Furthermore, while there is a large amount of longitudinal data related to the TST and its predictive value, there are only a few studies of IGRA, particularly in children, still remain relatively limited.25

**Discordance Between TST and IGRA Results**

Many pediatric TB experts use the TST and IGRA in parallel with the aim of increasing sensitivity, but this can be complicated by contradictory results. The underlying mechanisms of this discordance remain uncertain. In most pediatric studies, the number of children with a TST+/IGRA− result constellation is far greater than that with a TST−/IGRA+ constellation. Some authors contend that TST+/IGRA− discordance primarily results from prior BCG immunization or “immune sensitization” induced by exposure to NTM, and that it should thus be interpreted as a false-positive TST result (assuming the IGRA is true-negative as a result of greater specificity). However, the evidence to support this conjecture is limited. There is no doubt that BCG immunization can produce (“false”) positive TST results. However, an analysis of published data that included more than 240,000 subjects vaccinated with BCG in infancy found that, overall, only 8.5% had a positive TST result (here defined as ≥10 mm induration) that was attributable to the vaccine, while only 1% were TST-positive when tested 10 years or more after vaccination with BCG.29 Therefore, BCG immunization alone cannot account for the large proportion of children with TST+/IGRA− discordance reported by the majority of pediatric studies, which typically ranges between 10% and 40% of the study populations.6,26,32–34 There is also no doubt that NTM disease produces positive TST results in a substantial proportion of patients.40 Nevertheless, NTM disease is rare with most publications estimating the incidence in children to be less than 10 per 100,000 children per year.41,42 Therefore, NTM disease can also not account for the substantial proportion of individuals with TST+/IGRA− discordance in pediatric studies. The concept that environmental NTM exposure alone can induce memory T cells and thereby produce false-positive TST results is unproven, and the experimental data to support this theory are unconvincing.29 NTM are ubiquitous and the rate of isolation of NTM from environmental sources is identical worldwide.43 Despite this, the vast majority of individuals living in low TB prevalence countries are TST-negative.44,45 strongly suggesting that NTM exposure alone has limited impact on TST results.

The alternative explanation for TST+/IGRA− discordance is that the TST result is true-positive, while the IGRA result is false-negative (as a result of the latter having inferior sensitivity). Notably, recent data provide strong evidence that a significant proportion of individuals with TST+/IGRA− discordance are in fact latently infected with *M. tuberculosis*.36 Using whole blood assays with ESAT-6 and CFP-10 as stimulatory antigens, pro-inflammatory cytokine responses (including interferon-gamma, IP-10, TNF-alpha, IL-2, IL-12(p40) and IL-13 responses) were found to be significantly higher in a group of children with TST+/IGRA− discordance compared with a group of TB-uninfected children. This observation can not be explained by confounding caused by prior BCG vaccination, since neither ESAT-6 nor CFP-10 are expressed by any of the currently used BCG vaccine strains.

In the light of these uncertainties, in a child with a TST+/IGRA− result constellation, the possibility of latent TB cannot be reliably ruled out. Consequently, we, as well as other TB experts, strongly recommend that children with risk factors for TB infection (including known TB exposure or birth in a high TB prevalence country) with this discordant result constellation should be offered preventive treatment until such time that better immunodiagnostic assays that can reliably rule out latent infection with *M. tuberculosis* become available.47,48 This recommendation takes into account that children are at far greater risk of progression from LTBI to active TB over their lifetime compared with adults, and the fact that serious adverse events with standard preventive treatment (ie, isoniazid alone or in combination with rifampicin) are very rare in children.

**Molecular Assays for Tuberculosis**

Currently, there are a considerable number of commercial polymerase chain reaction (PCR)-based assays available for the detection of *M. tuberculosis*, some of which incorporate testing for the presence of drug resistance genes, including the COBAS TaqMan MTB (Roche Diagnostics, Basel, Switzerland), the ProbeTec ET Direct TB (Becton Dickinson, Franklin Lake, NJ), the FluoroType MTB (Hain Lifeciences, Nehern, Germany), the m2000 RealTime MTB (Abbott Laboratories, North Chicago, IL) and the Xpert MTB/RIF assay (Cepheid, Sunny Vale, CA). Following the official endorsement by the WHO in 2010, attention has focused on the latter assay. By September 2014, a total of 3553 Xpert instruments and almost 9 million Xpert MTB/RIF cartridges had been procured by countries eligible for concessional pricing (ie, low- and medium-resource countries; see <http://who.int/tb/laboratory/GeneXpert_rollout_large.png>).

The Xpert MTB/RIF assay is based on a qualitative, nested real-time PCR that allows *M. tuberculosis* complex to be directly detected in clinical samples, and can simultaneously detect mutations in the *rpoB* gene associated with rifampicin resistance. The assay has a number of advantages: it can be operated by personnel with minimal training, the sample preparation required is minimal, and the test result is available within 2 hours once the assay is initiated.49 However, disadvantages include the cost of the cartridges (currently $9.98 at concessional pricing), the need for a laboratory infrastructure with reliable power supply and air conditioning, and the need for regular maintenance of the analytical instruments, all of which pose considerable challenges in low-resource settings. Also, the assay cannot distinguish between live and dead *M. tuberculosis*, and can
therefore not be used to confirm treatment success or failure, or for the identification of relapse. The cost-effectiveness of the assay remains uncertain, but it appears obvious that its implementation and scale-up in countries with an annual health expenditure of $10–20 per capita represents a major challenge.49

A meta-analysis of early studies assessing the performance of the Xpert MTB/RIF assay reported that in pulmonary TB, the overall pooled sensitivity of the assay was 90.4% (95% confidence interval: 89.2–91.4), with a pooled specificity of 98.4% (98.0–98.7).50 However, disappointing, in smear-negative pulmonary TB (which applies to the majority of children) the pooled sensitivity was only 75.0%. It is therefore not surprising that studies in children with pulmonary TB have generally reported sensitivities ranging between 65% and 76%, although universally with specificities above 98%.51–53 While this yield represents a 2- to 3-fold improvement over sputum smear microscopy, these data also highlight that a quarter to one-third of pediatric cases would elude microbiological confirmation if the Xpert MTB/RIF assay was used as a replacement for mycobacterial cultures, which currently remain the gold standard in active TB.

Availability and Current Use of Immunological and Molecular Assays

Data on the availability and use of immunological and molecular assays in the routine clinical setting are surprisingly limited. A recent survey provides some insight into the current European landscape.54 The vast majority (93.6%) of participating European TB experts (based in 31 different countries) had access to PCR-based TB assays. Approximately two-thirds had access to commercial and the remaining one-third to non-commercial (in-house) PCR-based assays. Interestingly, the survey found that a large proportion of participants had used the Xpert MTB/RIF assay for the analysis of a variety of nonrespiratory samples (including pleural fluid, gastric aspirate fluid, cerebrospinal fluid, stool samples and blood/serum) despite the assay having been optimized and solely being licensed for the analysis of sputum samples.49 Although increasing data suggest that the Xpert MTB/RIF assay has relatively high sensitivity with cerebrospinal fluid and biopsy samples, recent reports highlight the assay’s suboptimal performance with stool samples, and pleural, peritoneal and joint fluids.54,55 Furthermore, the survey of European TB experts found that IGRA were also widely available across Europe, and that far more had access to the QFT-GIT than to the T-SPOT.TB assay (84.7% vs. 52.2%, respectively). This may reflect the practical difficulties of integrating an ELISPOT-based assay, such as the T-SPOT.TB assay, into the routine diagnostic laboratory setting.

Novel Methods for the Diagnosis of Tuberculosis

A detailed account of the current stage of development of novel TB diagnostics is outside the scope of this review, but can be found in a recent UNITAID report.56 Table, Supplemental Digital Content 1, http://links.lww.com/INF/C183 provides an overview of existing tests for TB, tests that are currently in development for commercial use, and diagnostic methods that are currently only available in the research setting. The requirements for new diagnostic tools for TB and the pathways of progression from the proof-of-principle stage to adoption in the clinical setting and in public health programs are outlined elsewhere.57

The SSI is currently developing a new skin test, the C-Tb test, which is based on recombinant ESAT-6 and CFP-10 and therefore likely to achieve greater specificity than the TST. A phase I trial (TESEC-01) shows that the test is well tolerated and that adverse events are rare.58 Data from the phase I dose finding trial (adults with confirmed and probable active TB; n = 38) and the phase II trial (healthy, BCG-vaccinated adults; n = 151) suggest that the C-Tb test has greater specificity (>95%) than the TST, but only limited sensitivity in active TB (17/24 individuals tested with a dose of 0.1 μg of C-Tb positive at the 5 mm induration cut-off; 71%).59 The results of a follow-on study (TESEC-04), which included 251 participants, 100 of which were HIV co-infected, will soon be available (personal communication; Dr Søren Tetens Hoff, SSI). Further studies (TESEC-05/46/07) in more than 3000 participants (including 722 children) have concluded recently (personal communication; STH). These preliminary results indicate that the C-Tb test will likely have greater specificity than the TST, but will have similar issues in relation to the subjectivity of the test result reading and interpretation. Nevertheless, the key advantages of the C-Tb test are that it can be performed at the primary care/community level without the need for laboratory infrastructure, that healthcare professionals are familiar with the test method (as it is identical to the TST), and that the anticipated costs are lower than those of IGRA-based testing.

The manufacturer of the QFT-GIT assay (Cellestis/Qiagen) has recently released the fourth generation of the assay, called Quantiferon-TB Gold Plus, in some European countries, with releases in further countries planned for the second half of 2015. As of March 2015, the FDA approval for the assay is still pending. The assay comprises 4 tubes (2 antigen-stimulated tubes, 1 positive and 1 negative control tube). According to the package insert, the antigen-stimulated tubes contain only ESAT-6 and CFP-10, while TB 7.7—which forms part of the current version of the assay—has been removed. The manufacturer claims that the performance of the first antigen-stimulated tube will be similar to the existing assay, while the second tube will primarily determine “CD8+ T cell responses,” with the aim of improving assay sensitivity in patients with active TB. Currently, there are no peer-reviewed publications on this new assay. Data included in the package insert suggest that the increase in assay sensitivity resulting from inclusion of the second antigen-stimulated tube will be marginal; in the evaluation studies, which included 174 individuals with culture-confirmed TB, the overall increase in assay sensitivity (compared with use of the first tube alone) resulting from the inclusion of the second tube was only 1.7%. The package insert and product presentations indicate that the assay has not been validated in children. An additional challenge for use of the assay in the pediatric setting is that the assay requires a larger blood volume (4 mL, instead of 3 mL for the QFT-GIT).

The current commercially available TB antibody-based (serological) tests have highly variable sensitivity and specificity, and the WHO therefore advises against their use.60 Notably, the WHO has encouraged the development of more robust antibody-based assays, and promising new multiplexed assays are currently in development.

Recent studies using a variety of flow cytometric methods for the detection of M. tuberculosis-specific T cells have also shown considerable promise. Data from 1 study in adults suggest that analysis of polyfunctional T cells (ie, T cells producing more than 1 cytokine) may allow the distinction between LTBI and active TB based on a blood test alone.61 A more recent study in children highlights the diagnostic potential of T cell activation marker analysis (based on CD27).62 However, the existing data for both assays are currently limited, and translation of these methods into a routine diagnostic setting in the near future appears challenging considering their current cost and complexity. In contrast, assays based on the detection of cytokine response signatures in supernatants following stimulation with M. tuberculosis-specific antigens (ie, based on similar principles as IGRA) are more likely to be translated into the routine diagnostic setting, as this can be achieved with much simpler and more robust methods. A small number of studies provide promising data suggesting that these assays may also enable the distinction between LTBI and active TB.63–65
A recent high-profile publication highlights the potential of using host RNA expression for the diagnosis of TB. The investigators identified a 51-transcript signature that achieved a sensitivity of 83% and specificity of 84%. However, this approach currently requires complex technology and it is uncertain whether this method can be down-scaled for use in a routine diagnostic setting.

Assays based on the detection of lipoarabinomannan (LAM) in sputum or in urine have been extensively evaluated in the research setting. Recently a commercial test, the Determine TB LAM Ag (Alere, Waltham, MA), has become available. LAM-based urine tests generally have insufficient sensitivity (≤50–60%) to be used as rule-out tests, but perform marginally better in HIV-infected patients with low CD4 T cell counts. Adenosine deaminase-based assays can only be used for the diagnosis of certain forms of extrapulmonary TB (TB meningitis, pleuritis, pericarditis and ascites), and have been shown to have limited sensitivity (typically ≤50–70%).

The recent detection of characteristic volatile organic compounds in the breath of patients with pulmonary TB has opened promising new avenues. Its noninvasiveness makes this approach attractive, but it is likely that this method will require relatively expensive instruments and perform worse in individuals with paucibacillary disease (including the majority of children). Also, volatile organic compound-based tests will not have the ability to detect extrapulmonary TB.

**DIRECTIONS FOR FUTURE RESEARCH**

This review highlights that despite considerable advances in recent years, the search for TB tests with better performance characteristics must continue. Existing diagnostic tests have suboptimal sensitivity, and generally perform worse in children compared with adults. Novel tests should particularly aim to achieve greater sensitivity, and would ideally be breath-, blood- or urine-based, as these samples can be obtained relatively easily from children. To be useful in settings in which childhood TB is most prevalent, tests should ideally be point-of-care assays, without the need for an existing laboratory infrastructure. Furthermore, a test that can reliably distinguish between LTBI and active TB is highly desirable, as this would help to guide management decisions. Future tests for LTBI should also have a better predictive value for the development of active TB, given that the predictive values of both TST and IGRA are comparatively low. As new tests are developed, it is crucially important that their performance characteristics are determined in sufficiently large studies in children, in light of the fact that existing tests almost universally perform worse in this patient group. Finally, further research is needed to determine how existing diagnostic approaches, in particular the use of the Xpert MTB/RIF assay in the routine clinical setting, impact on patient care and ultimately outcomes.

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