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Latent *Mycobacterium tuberculosis* Infection and Interferon-Gamma Release Assays

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ABSTRACT The identification of individuals with latent tuberculosis infection (LTBI) is useful for both fundamental understanding of the pathogenesis of disease and for clinical and public health interventions (i.e., to prevent progression to disease). Basic research suggests there is a pathogenetic continuum from exposure to infection to disease, and individuals may advance or reverse positions within the spectrum, depending on changes in the host immunity. Unfortunately, there is no diagnostic test that resolves the various stages within the spectrum of *Mycobacterium tuberculosis* infection. Two main immune-based approaches are currently used for identification of LTBI: the tuberculin skin test (TST) and the interferon-gamma release assay (IGRA). TST can use either the conventional purified protein derivative or more specific antigens. Extensive research suggests that both TST and IGRA represent indirect markers of *M. tuberculosis* exposure and indicates a cellular immune response to *M. tuberculosis*. The imperfect concordance between these two tests suggests that neither test is perfect, presumably due to both technical and biological reasons. Neither test can accurately differentiate between LTBI and active TB. Both IGRA and TST have low sensitivity in a variety of immunocompromised populations. Cohort studies have shown that both TST and IGRA have low predictive value for progression from infection to active TB. For fundamental applications, basic research is necessary to identify those at highest risk of disease with a positive TST and/or IGRA. For clinical applications, the identification of such biomarkers can help prioritize efforts to interrupt progression to disease through preventive therapy.

INTRODUCTION

Diagnosis and treatment of latent tuberculosis infection (LTBI) is one of the interventions recommended by the World Health Organization (WHO) to end the TB epidemic worldwide and is one of the elements of the post-2015 End TB Strategy (1). While several high-income countries, notably the United States and Canada, have implemented and scaled up programs to detect and treat LTBI, developing countries have mostly focused on active TB disease control, a much bigger priority in these settings.

In high-income countries, guidelines from agencies such as the U.S. Centers for Disease Control and Prevention (CDC) (2), Canadian Tuberculosis Standards (3), and the United Kingdom's National Institute for Health and Care Excellence (4) provide recommendations on LTBI management. For high-TB-burden countries, the WHO guidelines for the programmatic management of LTBI provide a blueprint for implementing targeted LTBI diagnosis and treatment, specifically in key affected populations such as people living with HIV/AIDS, adult and child contacts

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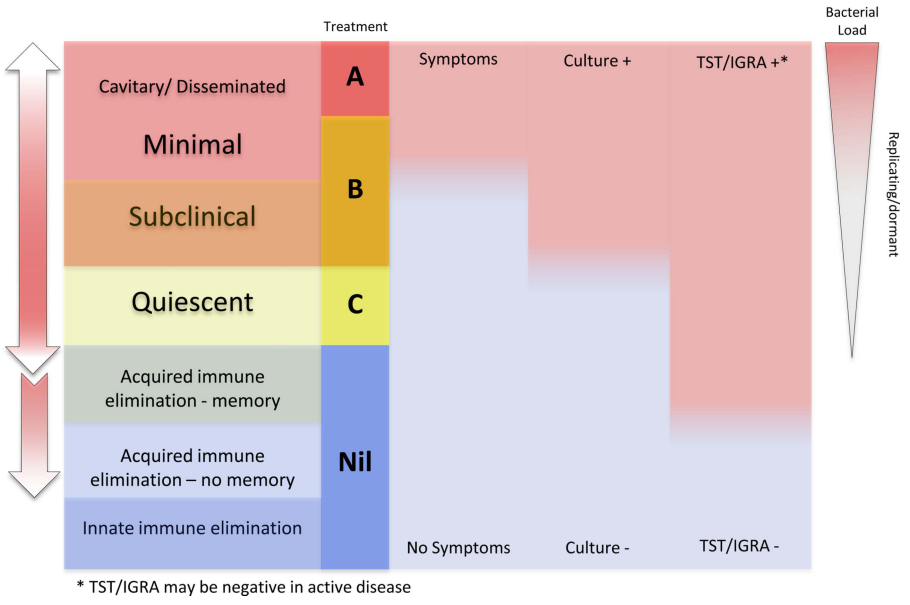
of pulmonary TB cases, patients initiating anti-tumor necrosis factor therapy, patients with end-stage renal failure, patients preparing for organ or hematologic transplantation, and patients with silicosis (5, 6). These populations are at high risk of TB exposure or at high risk of progressing from latency to active TB disease. With preventive therapy, it is possible to prevent the future occurrence of active TB disease (7), and a variety of drug regimens are WHO-endorsed for LTBI (6).

THE SPECTRUM OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION

In a majority of individuals who inhale *M. tuberculosis* bacilli, the infection is eliminated by innate immune responses or subsequently contained by poorly understood host defenses, and infection remains latent. Basic research suggests there is a continuum from exposure to infection, and infection to disease, and individuals in each of these classes can transition, either advance or reverse positions, depending on modulators of host immunity (8, 9). Figure 1, from Esmail, Barry, and Wilkinson, provides a helpful illustration of this proposed spectrum of TB (9). However, for clinical and public health decisions, TB is simplistically and pragmatically separated into three groups: (i) those with no evidence of infection, (ii) those with LTBI, and (iii) those with TB disease. In some populations, especially children, these simplistic classifications may be inadequate and misleading (10).

Although latency and active (i.e., symptomatic, infectious) TB disease are likely part of the same dynamic

FIGURE 1 A proposed framework for considering tuberculosis (TB) infection as a spectrum. In this model, from Esmail, Barry, and Wilkinson, after initial exposure, TB bacteria can be eliminated by innate immune mechanisms. Once infection is established and an acquired, adaptive immune response has been generated, interferon-gamma release assay (IGRA) or tuberculin skin test (TST) might become positive. Infection can be eliminated by the acquired immune response, but if antigen-specific effector T-cell memory persists, TST or IGRA might remain positive, even though infection is cleared. Over time, T-cell memory responses can wane, resulting in TST or IGRA reversions. If *M. tuberculosis* is controlled but not eliminated by the acquired immune response, the individual might enter a state of quiescent infection, in which both symptoms and culturable bacilli are absent and with a greater proportion of bacilli in a dormant rather than replicative state. Immunosuppression (e.g., HIV or drugs such as tumor necrosis factor blockers) during this state might lead to rapid progression to active disease. If bacilli are grown on culture and symptoms and signs are absent, this might be a subclinical state. If bacilli are grown on culture and symptoms appear, then this reflects active TB disease (which can range from smear-negative TB to advanced cavitary/miliary TB). (Reproduced from reference 9 with permission.)



spectrum (8, 9, 11), people with LTBI are typically considered to be asymptomatic and not infectious to others. However, people classified as having LTBI may harbor viable *M. tuberculosis* bacilli that can reactivate later, causing active TB disease. Studies suggest that 5 to 15% of individuals recently infected with *M. tuberculosis* progress rapidly (within 2 years) to active disease (12), whereas the remainder are considered to have LTBI and retain a persistent risk of reactivation (13). Identification and treatment of people with LTBI can help us understand the pathogenesis of disease, support ongoing efforts to develop new TB vaccines, and reduce the risk of development of disease via preventive therapy (also called chemoprophylaxis) (7, 14, 15).

TESTING METHODS FOR LTBI

The primary purpose of LTBI screening is to identify people who have evidence of *M. tuberculosis* infection and are at increased risk for the development of active TB. A key guiding principle is that only those who would benefit from treatment should be tested. This translates to the well-known dictum, "a decision to test should presuppose a decision to treat if the test is positive" (16).

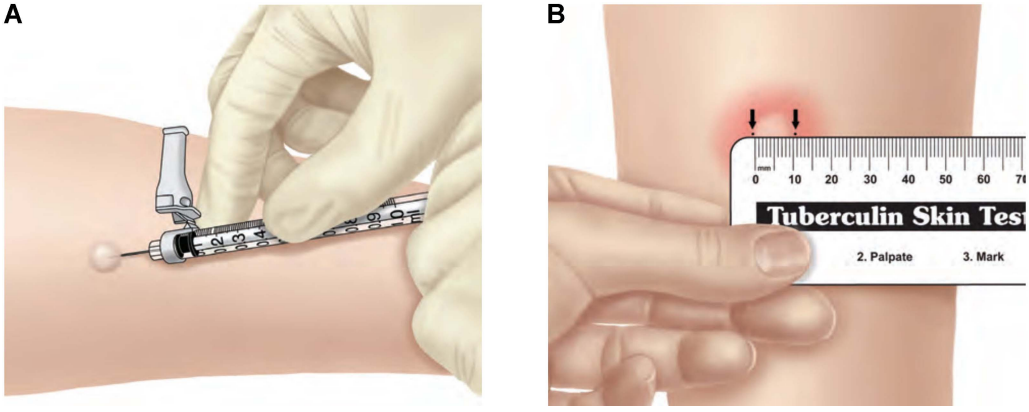
In general, testing for LTBI is indicated when the risk of development of disease from latent infection (if present) is increased; examples include likely recent infection (e.g., close contact with a TB patient) or decreased capacity to contain latent infection (e.g., because of immunosuppression). In contrast, screening for LTBI in people who are healthy and have a low risk of progressing to active disease is considered inappropriate, since the positive predictive value of LTBI testing for development of clinical disease is low and the risks of LTBI treatment (e.g., serious hepatotoxicity) can outweigh any potential benefits (7).

With regard to acceptable modalities of LTBI diagnosis, the WHO guidelines recommend that either a tuberculin skin test (TST) or an interferon-gamma release assay (IGRA) be used to detect LTBI in high-income and upper middle-income countries with estimated TB incidences less than 100 per 100,000 population (6). TST is preferred and IGRA should not replace TST in low- and middle-income countries whose TB incidence is ≥100 per 100,000 population (5, 6). This is primarily because IGRAs are more expensive to implement in such settings and do not add much more value compared to TST. In high-income countries, IGRAs are now quite widely used, although they have yet to replace TST entirely.

PURIFIED PROTEIN DERIVATIVE (PPD)-BASED TST

In many settings, the century-old TST, using PPD as the antigen, continues to be the frontline test for LTBI and, thus, the main driver of the LTBI preventive therapy. The TST is usually performed using the Mantoux method (17, 18) (Fig. 2). This consists of the intradermal injection of 5 tuberculin units (5 TU) of PPD-S or 2 TU of PPD RT23 (16). In a person with intact cellular immunity to these antigens, a delayed-type hypersensitivity reaction will occur within 48 to 72 hours. This delayed-type hypersensitivity reaction will cause erythema (red-

FIGURE 2 How to **(A)** administer and **(B)** read the tuberculin skin test (TST). TST involves an intradermal injection of 5 tuberculin units (5-TU) of PPD-S (purified protein derivative) or 2 TU of PPD RT23. A delayed-type hypersensitivity reaction might occur within 48 to 72 hours. This reaction will cause erythema (redness) and induration of the skin at the injection site. Only the transverse induration is measured as shown above and interpreted using risk-stratified cut-offs. (Adapted from reference 18.)



ness) and induration of the skin at the injection site. Only the transverse induration is measured (as millimeters of induration) and interpreted using risk-stratified cut-offs (14, 18). PPD administration and reading require training and skill. It is important to note that cellular immunity to PPD antigens can sometimes reflect exposure to similar antigens from environmental, nontuberculous mycobacteria or bacillus Calmette-Guérin (BCG) vaccination or to a previous infection that has been cleared (through immunological mechanisms or treatment) (19).

Interpretation of a TST result is not simple and involves much more than just the size of the induration. A TST result should be interpreted with the probability of prior infection in mind and the likely risk of disease if the person were truly infected (i.e., predictive value) (20). Menzies and coworkers have developed a user-friendly, online, interactive calculator—the Online TST/IGRA Interpreter (Version 3.0, www.tstin3d.com)—that incorporates all these dimensions (20). This risk calculator computes the probability of active TB development, given a TST or IGRA result, and accounts for other risk factors (e.g., history of contact or HIV), as well as BCG vaccination. The calculator also computes the risk of serious adverse events (e.g., hepatotoxicity) due to LTBI treatment.

While the TST has several advantages in low-resource settings, including low reagent cost, no hardware costs, limited skill requirement, and no requirement for laboratories, it does suffer from two big limitations: specificity of PPD-based TST is compromised by late (i.e., after the first year of life) or repeated BCG vaccination (i.e., boosters) and, to a limited extent, by exposure to nontuberculous mycobacteria (19). The BCG World Atlas is available (www.bcgatlas.org) to provide information on when and how many times countries give BCG (21). The second limitation is the limited predictive value for TB disease (15). In other words, a majority of individuals with positive TST results do not progress to active TB disease, so overtreatment is inevitable, since there is no way to know which individual with a positive TST result will actually benefit from LTBI therapy. The TST is also known to have limitations in reproducibility, and challenges such as interreader variability, boosting, conversions, and reversions are well documented (22). TST also has operational drawbacks, including the need for the patient to return for the reading.

IGRAS

IGRAs are *ex vivo* blood tests of T-cell immune response; they measure T-cell release of interferon-gamma (IFN-gamma), an inflammatory cytokine, following stimulation by antigens specific to the *M. tuberculosis* complex (with the exception of BCG vaccines and several exotic species, such as *Mycobacterium microti* and the Dassie bacillus). These antigens include early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), both encoded by genes located within the region of difference 1 locus of the *M. tuberculosis* genome (23, 24). They are considered more specific for *M. tuberculosis* than PPD because they are not produced by BCG vaccine strains, and only a few species of nontuberculous mycobacteria have been shown to produce these antigens (*Mycobacterium marinum*, *Mycobacterium kansasii*, *Mycobacterium szulgai*, and *Mycobacterium flavescens* [25]). There is some evidence of cross-reactivity between ESAT-6 and CFP-10 of *M. tuberculosis* and *Mycobacterium leprae* (26, 27), but the clinical significance of this in leprosy and TB-endemic countries (e.g., India and Brazil) is poorly understood and researched.

Two commercial IGRAs are available in many countries: the QuantiFERON-TB (QFT) Gold In-Tube assay (Qiagen, Valencia, CA) and the T-SPOT.TB assay (Oxford Immunotec, Abingdon, United Kingdom). Both tests are approved by the U.S. Food and Drug Administration (FDA), Health Canada, and Conformité Européenne marked for use in Europe. Figure 3 provides an overview of the immunological basis of commercial IGRAs (28).

The QuantiFERON technology has been through several iterations, with the first version using PPD as the stimulating antigen. The current QFT-Gold In-Tube assay is an enzyme-linked immunosorbent assay-based, whole-blood test that uses peptides from ESAT-6 and CFP-10 as well as peptides from TB7.7 [Rv2654c] (not a region of difference 1 antigen) in an in-tube format in which peptides are coated to the inner surface of the tubes into which venous whole blood is drawn. After incubation, an enzyme-linked immunosorbent assay is conducted to quantify the amount of IFN-gamma in international units per milliliter produced in the antigen tubes compared to the control tubes. An individual is considered positive for *M. tuberculosis* infection if the IFN-gamma response to TB antigens is above the test cut-off (after subtracting the background IFN-gamma response in the negative control).

QuantiFERON-TB Gold-Plus (QFT-Plus) is the next-generation IGRA launched by Qiagen in 2015. QFT-Plus uses two TB antigen tubes (TB1 and TB2). Both antigen tubes include peptides from ESAT-6 and CFP-10. While peptides in TB1 are designed to elicit an IFN-

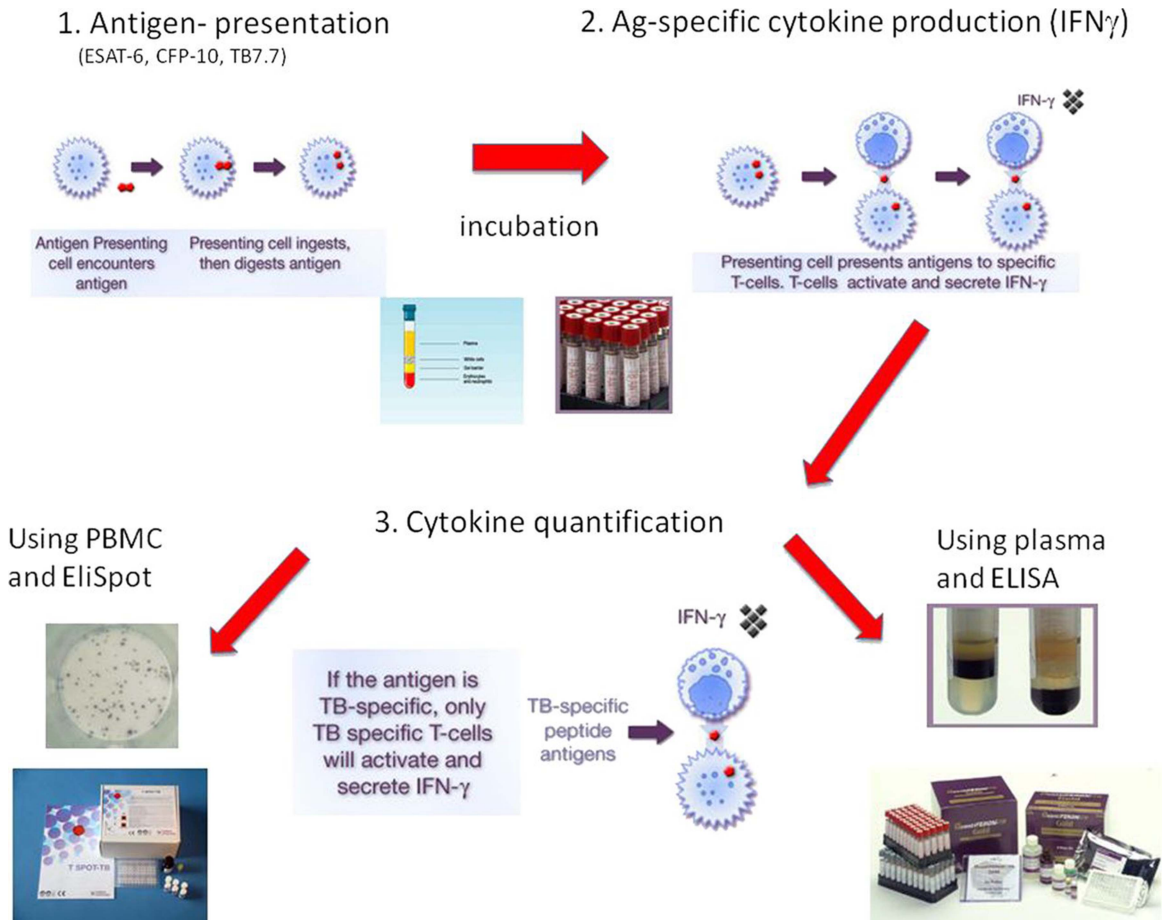


FIGURE 3 Immunological principles that underlie the existing, commercial interferon-gamma release assays. IFN-γ, interferon-gamma; PBMC, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay. (Reproduced from reference 28 with permission.)

gamma response from CD4+ helper T cells, TB2 contains additional peptides to elicit a response from CD8+ cytotoxic T cells. The aim is to increase the assay sensitivity. The test is interpreted as positive when either antigen tube result is positive. Published data on this newer assay are limited (29), but studies are ongoing. There is no policy guidance on QFT-Plus yet.

T-SPOT.TB is an enzyme-linked immunospot assay performed on separated and counted peripheral blood mononuclear cells that are incubated with ESAT-6 and CFP-10 peptides. The result is reported as the number of IFN-gamma-producing T cells (spot-forming cells). An individual is considered positive for *M. tuberculosis* infection if the spot counts in the TB antigen wells exceed a specific threshold relative to the negative control wells.

What is the evidence on IGRAs? A decade ago, early data raised the hope that the TST could be replaced by an *in vitro* assay with better performance (30). A decade later, after a large number of research studies, evidence shows that both TST and IGRA are acceptable but imperfect tests for LTBI screening (15). Table 1 provides a comparison of TST and IGRA (31). Both tests are indirect markers of *M. tuberculosis* exposure, and neither test is able to accurately differentiate between LTBI and active TB (32) or to resolve the various phases within the *M. tuberculosis* infection continuum (8, 15).

Studies show that both TST and IGRA cannot distinguish individuals who have successfully cleared *M. tuberculosis* infection (i.e., no longer need therapy) from those who have true infection (which is amenable to therapy) (33). This inability to differentiate results in overtreatment with increased costs and adverse events. Both TST and IGRA have reduced sensitivity in immunocompromised patients, particularly in those with a severe immune depression, and have low predictive

TABLE 1 A comparison of available diagnostics for latent TB infection[a]

Characteristic	PPD-based tuberculin skin tests	Newer, specific skin tests (under development or validation)	Interferon-gamma release assays
Examples of products in the category	Tubersol, Aplisol, PPD RT23	C-Tb, Diaskintest	QuantiFERON-TB Gold In-Tube; T-SPOT.TB
Testing format	Intradermal skin test (*in vivo*)	Intradermal skin test (*in vivo*)	*Ex vivo* assay (ELISA or ELISPOT)
Antigens used	Purified protein derivative	ESAT-6 and CFP-10	ESAT-6 and CFP-10
Intended use	Screening for LTBI	Screening for LTBI	Screening for LTBI
Sensitivity	High	Modest	Modest
Sensitivity in immunocompromised populations	Reduced	Reduced	Reduced
Specificity	Modest	High	High
Impact of BCG on specificity	High (when BCG is given after infancy or multiple times)	None	None
Ability to distinguish latent from active TB	Low	Low	Low
Ability to predict progression to active TB disease	Modest	Unknown (but likely to be modest based on indirect evidence from IGRAs)	Modest
Ability to resolve the various stages within the spectrum of *M. tuberculosis* infection	Low	Low	Low
Reagent costs	Low	Unknown (but likely to be low based on indirect evidence from PPD-based TST)	High
Requirement for laboratories	No	No	Yes

[a]Data from reference 31. BCG, bacillus Calmette-Guérin; CFP-10, culture filtrate protein; ELISA, enzyme-linked immunosorbent assay; ESAT-6, early secreted antigen target; IGRA, interferon-gamma release assay; LTBI, latent tuberculosis infection; PPD, purified protein derivative.

value for progression to active TB (15, 34). As in the case of PPD-based TST, a majority of individuals (i.e., over 95%) with positive IGRA results do not progress to active TB disease. This has been seen in several longitudinal studies and reviewed systematically by Rangaka and colleagues (34) and by WHO (5). Compared to the PPD-based TST, IGRAs have overcome the limited specificity problem, because BCG vaccination does not impact the test results, but they have not overcome the problem of limited predictive value (34).

Another emerging concern with IGRAs is their highly dynamic nature, with inconsistent results and high rates of conversions and reversions, when repeated tests are performed. Some of this dynamism could reflect transitions within the LTBI spectrum, while some is likely related to poor assay reproducibility. Several serial testing studies of low-risk health care workers have revealed higher false conversion rates with IGRAs than with TSTs (35–38). Reproducibility studies have identified various sources of variability that contribute to nonreproducible results, and these studies have been systematically reviewed elsewhere (39). As reviewed by Banaei and colleagues, sources of variability can be broadly classified as preanalytical, analytical, postanalytical, manufacturing, and immunological (40). Figure 4 provides an overview of the major sources of variation with the QFT assay (15, 40). Similar challenges also affect the reproducibility of the T-SPOT.TB assay.

It is therefore important to note that both TSTs and IGRAs have reproducibility challenges, and dichotomous cut-offs are inadequate for interpretation. Further, extensive efforts need to be made to ensure adequate training and standardization. Table 2 lists suggestions to better standardize IGRAs and reduce the amount of test variability (40). When IGRAs are used for serial testing of health care workers, simplistic cut-offs (e.g., change from negative to positive) should not be used, because this results in very high conversion rates (41). There is no clear consensus on the best cut-off to use for serial testing with IGRAs (42), and many hospitals tend to repeat an IGRA test among low-risk individuals to check if the repeat test stays positive.

NEWER, SPECIFIC SKIN TESTS

These newer skin tests replace PPD with more specific antigens but in the same intradermal test format. C-Tb, a novel *M. tuberculosis*-specific skin test containing ESAT-6 and CFP-10 antigens, is one such new skin test, developed by Statens Serum Institut, Denmark (43, 44). Another product, Diaskintest (Generium Pharmaceutical, Moscow, Russia), is available commercially in

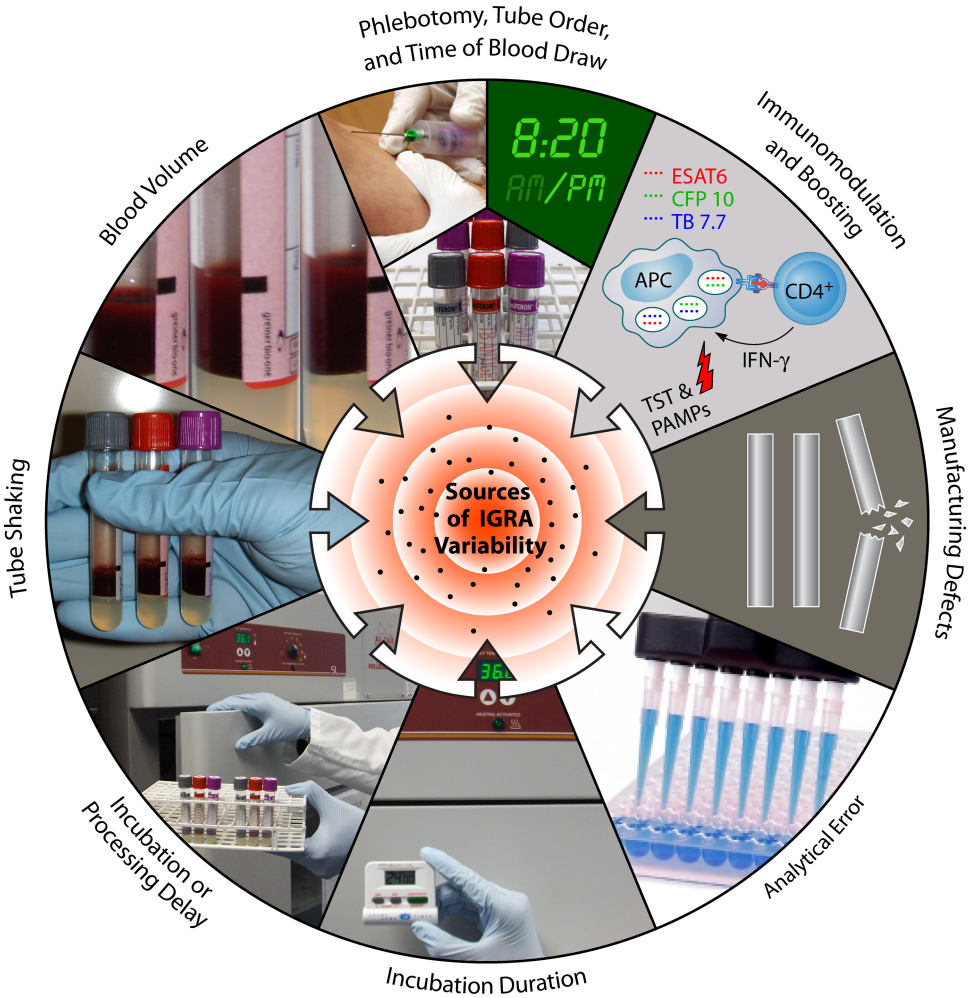


FIGURE 4 Sources of variability in the QuantiFERON-TB (QFT) Gold In-Tube assay. This graphic illustrates the sources of variability that affect the reproducibility of the QFT-Gold In-Tube assay. Variability can be due to preanalytical, analytical, postanalytical, manufacturing, and immunological factors. (Reproduced from reference 15 with permission.)

Russia, Ukraine, and Kazakhstan (45), and an ESAT-6-based skin test from China is in clinical trials (46). There is currently no policy guidance on these newer skin tests, because the evidence base is weak.

By substituting PPD with ESAT-6 and CFP-10, these newer skin tests appear to overcome the specificity limitations of the PPD-based TST (43, 44). But this improvement in specificity might come at the cost of reduced sensitivity. One recent trial from South Africa suggests that the sensitivity of PPD-based TSTs is comparable to that of QFT-Gold In-Tube but lower than that of PPD-based TSTs (47). In this trial, the sensitivity of all LTBI tests was compromised in immunosuppressed HIV-infected patients (47).

Although further validation is required, it appears that newer skin tests do offer higher specificity than PPD-based TSTs, but this might compromise sensitivity (Table 1) (31). While there are no data on the predictive value of the newer skin tests, it is highly likely that the predictive value will be modest, based on what is already known about IGRAs based on ESAT-6 and CFP10 peptides (34). Thus, when compared to the PPD-based TST, IGRAs and newer skin tests might offer some incremental advantages, primarily, improved specificity. However, if reagents can be produced at scale and at affordable prices, newer skin tests may help resolve PPD shortages that have been reported in many settings (48).

CONCLUSIONS

Both TSTs and IGRAs are now a part of the LTBI testing landscape, and current guidelines allow the use of both tools, although guidelines vary considerably across countries (49). There are situations where neither test is

TABLE 2 Some suggested approaches to reduce test variability with IGRAs[a]

Step during the assay	Suggestions for best practices
Disinfection	Standardize skin and tube septum disinfection, akin to that done for blood cultures.
Tube order	Standardize the order of the GFT-GIT tubes during phlebotomy per the package insert (in the order purge tube, nil tube, antigen tube, and mitogen tube).
Blood volume	Standardize blood volume drawn into the QFT-GIT tubes, particularly for the antigen tube. Filling the tubes up to the 1-ml mark is practical. Collecting blood using a syringe and transferring 1 ml to each of the tubes is more accurate.
Tube shaking	Standardize gentle shaking of the QFT-GIT tubes per the package insert. Avoid separate shaking of the nil and antigen tubes, because differential shaking can result in a false-positive or false-negative result.
Processing delay	Minimize delays in incubation of cells. For the QFT-GIT assay, this can be achieved by placing an incubator at the collection site or by using a portable incubator to transport the tubes from the clinic to the laboratory. Further studies are needed to determine whether the T-Cell Xtend reagent can prolong processing time for the T-SPOT assay.
Analytical error	Use automated ELISA and ELISPOT instruments to reduce analytical variability.
Manufacturing defect	Institute a quality assurance program to monitor positivity and indeterminate rates. When rates cross a preset threshold and persist, halt utilization of potentially faulty lots and alert the manufacturer.
Immune boosting	When a two-step testing procedure (TST followed by IGRA) is used, TST boosting of the IGRA result can be avoided by drawing the blood sample for IGRA within 72 h of TST placement.

[a]Data from reference 40. ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay; IGRA, interferon-gamma release assay; QFT-GIT, QuantiFERON-TB Gold In-Tube; TST, tuberculin skin test.

appropriate (e.g., diagnosis of active TB in adults), and situations where both tests may be necessary to detect *M. tuberculosis* infection (e.g., immunocompromised populations), since no single test is adequate. And there are situations where one test might offer clear advantages over the other. For example, IGRAs would be preferable to the TST in populations where BCG is given after infancy or given multiple times (19, 21). In contrast, TST is preferable to the IGRAs for serial testing of health care workers, because IGRAs produce high rates of conversions and reversions and are harder to interpret for occupational health programs (3, 41, 50).

Unfortunately, none of the available LTBI tests meet a big felt need in the TB field—a highly predictive test that can help target those who will benefit most from LTBI therapy. To develop such predictive tests, we need transformative research that will enable us to identify biomarkers or biosignatures that can resolve the LTBI spectrum (8) and help target those at highest risk of progressing to active disease (51). Some promising biomarkers have been identified, especially gene expression signatures (52), but much more validation work is required.

A target product profile for such a predictive LTBI test has been developed by the New Diagnostics Working Group of the Stop TB Partnership (53), FIND, and other partners, and recent TB diagnostics market analyses and projections (54) might also help increase industry and donor interest in research and development that will result in the development of such innovative products that make an impact. Ideally, a more predictive LTBI test will also serve as a marker of cure after LTBI therapy.

Until we have substantially improved tools for LTBI, to maximize the predictive value of existing tests, LTBI screening should be selectively used for those who are at sufficiently high risk of progressing to disease. Such high-risk individuals may be identifiable via multivariable risk prediction models that incorporate test results with traditional risk factors (e.g., using risk calculators such as www.tstin3d.com) and via serial testing to resolve underlying phenotypes (15). Needless to say, LTBI testing should be followed by adequate counseling to ensure completion of LTBI therapy.

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