

Interferon- γ release assay

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Abstract

Traditionally, tuberculosis (TB) has been viewed as having two distinct manifestations, known as TB infection (TBI) and TB disease. The spectrum, however, has recently been expanded to include the elimination of TBI with the help of innate and/or adaptive immunity, TBI, incipient and subclinical TB disease, and TB disease. Epidemiologically speaking, identifying individuals with TBI is critical since diagnosis and treatment of TBI are essential in controlling the TB burden. It is important to identify high-risk individuals with TBI who are more likely to progress to active TB disease. There are two diagnostic methods for identifying TBI. These include the conventional tuberculin skin test (TST) and interferon- γ release assay (IGRA). However, these methods are not the “gold standard”. Furthermore, all of these methods are indirect, relying on the host’s adaptive immune response to *Mycobacterium tuberculosis*-derived protein antigens. This review will describe the various tests for TBI, such as TST, IGRAs, newer skin and blood tests, methods for performing IGRAs, interpretation strategies, and limitations.

Key words: *Mycobacterium tuberculosis*, interferon- γ release assay, tuberculosis infection, tuberculin skin test.

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Introduction

Tuberculosis (TB) has been known to infect mankind since time immemorial and is caused by the *Mycobacterium tuberculosis* (M.tb) complex. The World Health Organization (WHO) Global TB Report 2023 stated that the incidence of TB cases in 2022 was 133 (95% uncertainty interval: 124-143) per 100,000 population. In absolute terms, the total number of symptomatic TB cases was 10.6 million. Most cases occurred in Southeast Asia (46%) and Africa (23%) [1]. The total number of deaths was 1.3 million. TB is traditionally described in two forms: TB disease and TB infection (TBI).

After the acquisition of infection by an immunocompetent person, the TB bacilli enter a latent state in 90% of cases. In immunocompetent individuals, the bacilli may undergo reactivation subsequently at a rate of 10% in their lifetime. TBI is defined as a state of persistent immune response to M.tb antigens without evidence of clinically manifested TB disease [2]. Patients with TBI may harbor live M.tb bacilli, but presently we do not have the diagnostic modalities to detect the live bacilli. Although the bacteria are metabolically active and dividing, a potent immune system controls the infection. The immunodiagnostic tests only reflect the persistent adaptive immune response to M.tb and not the true latent infection [3]. The Tuberculosis Network European Trials group consensus statement stated that the tuberculin skin test (TST) and interferon- γ release assay (IGRA) positivity indicate “lasting TB immune responses” and not necessarily “latent TBI”, and it is unknown how long the adaptive immune responses towards mycobacterial antigens persist

in the absence of live mycobacteria [3]. Approximately 1.7 billion people, or 23% of the world’s population were infected with M.tb, and approximately 80% of all TBI cases were from the WHO regions of Southeast Asia, Western Pacific, and Africa [4,5].

These large pools of infected people are the harbingers of future active TB disease. In a mathematical modeling study, Houben *et al.* reported that by 2035 and 2050, a pool of 1.7 billion latently infected people will generate 16.5 and 8.3 per 100,000 per year of active TB patients, respectively [5]. In a transmission-dynamic model, TB preventive therapy has a greater impact on overall TB incidence than most other single interventions [6]. A crucial step in TB control is to identify at-risk individuals with TBI who would benefit from TB preventive therapy. Currently, two diagnostic tests are in use, including TST and IGRA for diagnosing TBI.

Tuberculin skin test

In 1882, Robert Koch discovered the tubercle bacillus. It was named M.tb in 1886 [7]. Clemens von Pirquet, in 1907, developed tuberculin as a diagnostic agent for TB [8]. French physician Charles Mantoux subsequently developed the interpretation strategy for the TST [9]. A purified protein derivative (PPD) from the attenuated M.tb strain was used in the injection. The TST, also known as the Mantoux test, has been used for over a century to diagnose TBI. It involves injecting 0.1 mL of PPD intradermally into the volar aspect of the forearm. A special buffer, Tween-80, is employed to stabilize the solution. The diameter of the induration



is measured and recorded 48 to 72 hours later in mL. The test solution was standardized against batch RT-23 produced by the Statens Serum Institut (SSI) in Denmark. The TST is cost-effective and simple to implement in field settings, allowing for rapid testing of a large number of patients. However, the TST has limitations in terms of performance, sensitivity, and specificity [10]. The test lacks specificity as it may yield positive results in individuals previously vaccinated with *Bacillus Calmette-Guérin* (BCG) strains. Moreover, there is cross-reactivity with numerous nontuberculous mycobacterium (NTM) species [11]. The sensitivity is also suboptimal in individuals with HIV infection, particularly those with low CD4 counts and not on highly active antiretroviral therapy, within 10 weeks of TBI, lymphoma, live virus vaccination (measles, smallpox), immunosuppressive therapy, overwhelming TB (e.g., military TB), extreme age (newborns, elderly), and other infections. The prevalence of PPD reactivity in HIV-positive patients with CD4 counts below 400 lymphocytes/mm³ is 2.7%, compared to 10.0% in HIV-negative controls ($p < 0.001$) [12]. Furthermore, the TST may exhibit a booster effect, characterized by increased skin reactivity 1 week to 1 year after the initial negative test, attributed to an immunological recall phenomenon. Boosting is commonly noticed when the time interval between the first and second tests is between 1 and 5 weeks. It is less common within 48 hours or longer than 60 days [13]. A false negative test result may occur due to inadequate PPD storage and poor administration technique. Furthermore, the test necessitates two visits, and there is inter-reader and intra-reader variability. The window period between infection and the development of tuberculin positivity is 12 weeks.

Interferon- γ release assay

IGRA is an *in vitro* diagnostic assay. It uses early-secreted antigenic targets (ESAT-6), culture filtrate proteins (CFP-10), and TB7.7 antigens. Streeton *et al.*, in the late 1990s, developed a novel *in vitro* blood test that used PPD to stimulate T-cells within whole blood before detection of released interferon- γ (IFN- γ) [14]. They used this test for TBI detection. They measured IFN- γ (IU/mL) responses as % PPD/phytohaemagglutinin response ratios. Using the PPD response ratio of 15%, the sensitivity and specificity of the IFN- γ assay were 90% and 98%, respectively. This assay was later known as QuantiFERON-TB (Cellestis Inc., Victoria, Australia) and subsequently approved by the Food and Drug Administration (FDA), United States of America, in 2001 [15]. Besides PPD, the QuantiFERON-TB assay also used the *Mycobacterium avium* antigen. Besides these, there are mitogen tubes as positive controls (phytohaemagglutinin) and nil tubes containing saline as negative controls. The test found positive responses in 83% of those with active TB disease and 59% of the previously treated cases. Other categories that showed positive responses were untreated inactive disease (80%) and exposed but TST-negative group (43%). However, the PPD-based assay lacks specificity as the protein components are shared by *Mycobacterium bovis* (*M. bovis*) and NTM. Advancement in mycobacterial genomics, including the discovery of a region of difference 1 (RD-1) segment of the *M.tb* genome, has made PPD-based assays irrelevant [16]. The RD-1 is a 9.5-kb DNA fragment that is missing from all BCG sub-strains but found in all virulent laboratory and clinical strains of *M. bovis* and *M.tb*. [17].

Two antigenic targets localized to the RD-1 are the 6 kDa ESAT-6 and the 10 kDa CFP-10. Both of these antigenic targets are absent in BCG strains and the majority of NTM species except *M. kansasii*,

M. szulgai, *M. marinum*, *M. gastri*, *M. leprae*, and *M. flavescens* [18,19]. The TB7.7 antigen is encoded in RD-11 and is missing from the BCG strains as well as the most common environmental mycobacteria [20]. Following the identification of these RD-1-related antigens, ESAT-6 and CFP-10 replaced PPD, and the test was known as QuantiFERON-TB Gold (QFT-G) in 2005.

Two more versions of the QuantiFERON-TB assay were developed subsequently: the QuantiFERON Gold In-Tube (QFT-GIT) (Cellestis, Ltd., Victoria, Australia) assay and the QuantiFERON-TB Gold Plus (QFT-Plus) (QIAGEN, Carnegie, Australia) assay. The QFT-GIT assay used three antigens, including ESAT-6, CFP-10, and TB7.7. Antigen-coated vacutainer blood collection tubes are used. The QFT-GIT assay also uses a nil tube, a mitogen tube, and an antigen tube. The QFT-GIT assay became commercially available in 2007 [21]. The QFT-Plus assay is a newer version of QFT-GIT and was launched in 2015. The QFT-Plus assay is different from the earlier version because of the presence of two antigen tubes. TB antigen tube 2 (TB2) contains both long and short peptides of ESAT-6 and CFP-10. They are recognized by MHC classes I and II. They stimulate IFN- γ production by both CD4+ and CD8+ T-cells [22,23]. The TB antigen tube 1 (TB1) contains long peptides derived from ESAT-6 and CFP-10 and is recognized by MHC class II molecules for presentation to CD4+ T-cells [24]. Therefore, the QFT-Plus assay has better utility for immunosuppressed patients. Professor Lalvani's group in Oxford, United Kingdom, developed the enzyme-linked immunospot (ELISpot) assay [25,26]. The commercially approved test was marketed in 2004 as the T-SPOT.TB test (Oxford Immunotec Limited, Abingdon, United Kingdom). The QFT-GIT and QFT-Plus assays are enzyme-linked immunosorbent (ELISA)-based assays that measure the amount of IFN- γ released by lymphocytes in response to specific TB antigens. On the other hand, the T-SPOT.TB is an enzyme-linked immunospot assay that counts the number of IFN- γ -producing cells (spot-forming cells). T-SPOT.TB assay uses peripheral blood mononuclear cells (PBMCs), which are separated from peripheral blood and are stimulated by ESAT-6 and CFP-10. PBMCs are monocytes and lymphocytes. The T-SPOT.TB stimulates both CD4+ and CD8+ T-cells.

There are a few modifications to the T-SPOT.TB test. T-Cell Select is an automated sample preparation system that includes the automatic isolation of PBMCs [27]. The T-Cell Xtend reagent allows blood samples to be processed up to 36 hours after venepuncture without affecting the accuracy of the test [28]. The Wantai TB-IGRA was launched in 2011 by the Beijing Wantai Biological Pharmacy Enterprise [29]. This test is similar to the QFT-Plus assay. It includes a positive control, a negative control, and an antigen tube. WANTAI TB-IGRA uses a recombinant fusion protein of CFP-10 and ESAT-6, whereas QFT-Plus uses polypeptide antigens of CFP-10 and ESAT-6 [30]. The WHO recommends Beijing Wantai's TB-IGRA and Qiagen QFT-Plus assays [31].

Interferon- γ release assay efficacy

Mori *et al.* evaluated the two RD-1 region antigens, CFP-10 and ESAT-6, in the detection of TBI in BCG-vaccinated individuals on the QuantiFERON-TB platform [32]. They estimated the sensitivity and specificity in culture-confirmed *M.tb* and patients with no identified risk of exposure. The reported sensitivity and specificity were 89% and 98.1%, respectively, with a cut-off of 0.35. The specificity of the TST test in this study was 35.4%. In a systematic review, the pooled sensitivity of QFT-G, QFT-GIT, and T-SPOT.TB was 78% [95% confidence interval (CI) 73-82%], 70% (95% CI 63-78%), and 90% (95% CI 86-93%), respectively [33]. The pooled specificity of



both QuantiFERON tests among non-BCG-vaccinated and BCG-vaccinated participants was 99% (95% CI 98-100%) and 96% (95% CI 94-98%), respectively, whereas the pooled specificity of T-SPOT.TB assay was 93% (95% CI 86-100%). Therefore, IGRA is not affected by prior BCG vaccination status and the T-SPOT.TB test is more sensitive than other IGRA assays. The pooled sensitivity of TST was 77% (95% CI 71-82%), whereas the pooled specificity of TST in non-BCG-vaccinated participants was 97% (95% CI 95-99%). In another systematic review and meta-analysis [34], the pooled specificity of IGRA and TST was 98 to 100% and 88.7% (95% CI 84.6-92.0), respectively for the diagnosis of TBI. The pooled negative predictive value (NPV) for QFT-GIT and T-SPOT.TB was 99.8% (95% CI 99.4-100) and 97.8% (95% CI 94.5-99.4%), respectively. The positive predictive value (PPV) for developing active TB among subjects who had tested positive for TBI and refused preventive treatment was 2.3 to 3.3% for TST, 2.8 to 14.3% for QFT-GIT, and 3.3 to 10% for T-SPOT.TB. Therefore, IGRA showed higher specificity and a higher PPV compared to TST. The high NPV indicates that TST and IGRAs will reliably exclude progression to TB. The low PPV of IGRA and TST suggests that the number needed to be treated (NNT) to prevent a single case of TB is 25 to 33, with the caveat that there is 100% adherence to and effectiveness of the preventive therapy [35]. Two systematic reviews and meta-analyses had shown a higher sensitivity of QFT-Plus compared to that of QFT-GIT for detecting TBI [36,37]. The increased sensitivity might be due to the CD8+ T-cell immune responses in the TB2 tube. CD8+ T lymphocytes indicate exposure to *M.tb* pathogens in situations where there are few CD4+ T lymphocytes, such as HIV infection. Xu *et al.* compared QFT-Plus and QFT-GIT in immune-compromised individuals and reported a good agreement between the two assays (Cohen's κ of 0.859) [38]. Furthermore, QFT-Plus detected more cases in immune-compromised patients. The predictors of indeterminate results were lower lymphocyte counts, lower CD4+ T-cell and CD8+ T-cell absolute counts, and a lower CD4/CD8 ratio. They also reported the following thresholds that will reduce indeterminate results: absolute lymphocyte counts of $>1.15 \times 10^9$ cells, and absolute CD4+ T-cell counts of $>467.7 \times 10^6$ to 478.5×10^6 cells. Therefore, both QFT-Plus and QFT-GIT assays showed good agreement even in immune-compromised individuals. A meta-analysis showed higher IFN- γ production in TB2 than TB1 [39]. Venkatappa *et al.* compared and evaluated the agreement of test results for QFT-Plus with those of QFT-GIT, T-SPOT.TB, and the TST for the diagnosis of TBI [40]. They reported 94% agreement between QFT-Plus and QFT-GIT, 77% agreement between QFT-Plus and QFT-GIT with TST, 92% agreement between QFT-Plus and T-SPOT.TB, and 91% agreement between QFT-GIT and T-SPOT.TB, respectively. Gupta *et al.* found a modest increase in PPV with a higher quantitative IGRA assay [41]. The PPV was 3.0% for QFT-GIT ≥ 0.35 IU/mL and 3.6% for ≥ 4.00 IU/mL, whereas with T-SPOT.TB, it was 3.4% for ≥ 5 spots and 5.0% for ≥ 50 spots. The WHO consensus statement suggested the features of an optimal target product profile (TPP) of a test that should predict progression from TBI to active disease within 2 years after infection with a sensitivity and specificity of $\geq 90\%$ or at least $\geq 75\%$, a high PPV, and a low NNT [42]. Therefore, the available diagnostic assays for TBI are not ideal.

Interferon- γ release assay among immunocompromised patients

In HIV-infected individuals, IGRAs show better performance than TST in identifying TBI, as its sensitivity remains relatively

unimpaired in moderately advanced HIV infection, unlike TST [43,44]. T-SPOT.TB may have an advantage over TST and QFT-GIT in patients with low CD4 lymphocyte counts, as the T-SPOT.TB test functions independently of CD4 lymphocyte counts [45]. Cattamanchi *et al.*, in a systematic review and meta-analysis, assessed the role of IGRA in TBI among HIV-infected individuals [46]. They included 37 studies with a total of 5736 HIV-infected individuals. The pooled sensitivities for T-SPOT.TB and QFT-GIT were 72% (95% CI 62-81%) and 61% (95% CI 41-75%), respectively. Therefore, in immunocompromised individuals, T-SPOT.TB is more sensitive than IGRA. T-SPOT.TB retains sensitivity in immunosuppression, as this test requires an adequate number of PBMCs in each test well. Table 1 shows the difference between IGRA and TST.

Methods of performing interferon- γ release assay

Both TST and IGRA depend on the detection of cell-mediated immune responses to *M.tb* antigens. IGRA assays are based on the principle that patients infected with *M.tb* have primed T-cells. When whole blood is incubated with *M.tb* antigens, exposure of primed T-cells to *M. tb* antigens occurs, which leads to the production of IFN- γ . The released IFN- γ is detected by ELISA or ELISpot methods. The QFT-Plus assay uses the ELISA platform. The QFT-Plus assay uses four tubes: a nil tube, a mitogen tube, a TB1 antigen tube, and a TB2 antigen tube. The nil tube and mitogen tube are used as negative and positive control, respectively. The nil tube measures the background or circulating IFN- γ level in the absence of antigens. The mitogen tube uses phytohaemagglutinin as mitogen, which is a universal T-cell stimulus. It assesses the capacity of T-cells to produce IFN- γ upon stimulation with a mitogen. Both TB1 and TB2 tubes contain peptide antigens consisting of ESAT-6 and CFP-10. In the TB1 tube, the antigens stimulate CD4+T-helper lymphocytes. The TB2 tube contains an additional set of shorter peptides that also stimulate CD8+ cytotoxic T lymphocytes [47]. First, 4 mL of whole blood is collected by venepuncture into specialized blood collection tubes, namely the nil tube, the mitogen tube, and the two antigen tubes. Another option is to collect the blood into a lithium or heparinized vacutainer and subsequently transfer it into the four specialized tubes. Approximately 1 mL of blood was added to each of the nil, TB antigen, and mitogen tubes. Now the tube should be shaken for proper mixing of blood with the antigens dried on the wall. Samples should be sent to the referral laboratory within 16 hours. The samples in the reference laboratory are now incubated for 16 to 24 hours at 37°C [48]. After incubation, tubes should be centrifuged at 2000-3000 relative centrifugal force for 15 minutes, and the plasma should be separated. The released IFN- γ concentrations from lymphocytes are measured using an ELISA test. The levels of IFN- γ are measured in international units (IU) [49]. Plasma samples can be stored for up to 28 days at 2°C to 8°C or below -20°C for extended periods. The negative control or the nil tube must have an IFN- γ value of ≤ 8 IU/mL; otherwise, the test will be considered invalid. A high value in nil tube indicates high levels of circulating heterophile antibody or non-specific, circulating IFN- γ . For example, in patients with rheumatoid arthritis and systemic lupus erythematosus, the circulating IFN- γ levels are often elevated [19]. The positive control or the mitogen tube must have an IFN- γ value of ≥ 0.5 IU/mL higher than the nil tube. A low response in the mitogen tube indicates insufficient IFN- γ produc-



Table 1. Difference between interferon- γ release assay and tuberculin skin test.

Setting of the test	IGRA <i>In vitro</i>	TST <i>In vivo</i>
Basic principle	It measures adaptive immune response to mycobacterial tuberculosis specific antigens	Delayed-type hypersensitivity response to the intradermal inoculation of PPD
Antigens	ESAT-6, CFP-10, and TB7.7	PPD which contains more than 200 antigens
Antigenic cross-reactivity and false-positivity	<i>Mycobacterium bovis</i> and NTM such as <i>M. kansasii</i> , <i>M. szulgai</i> , <i>M. marinum</i> , <i>M. gastri</i> , <i>M. leprae</i> , and <i>M. flavescens</i>	<i>Mycobacterium bovis</i> , BCG and many NTM
Instrumentation and laboratory infrastructure	ELISA or ELISpot based test so instrumentation and sophisticated laboratory infrastructure are required	Do not need sophisticated instrument and can be done in field condition
Interpretation	Laboratory-based	Operator-based
Ease of use	Can be used in specialized laboratory	Can be applied in field condition
Outcome	Measures the levels of IFN- γ	Measures induration
Number of visit	Single patient visit	Two patient visits
Boosting	TST-induced “boosting” of IGRA responses occurs after 3 days and may persist for several months [87]	Boosting occurs after repeated testing
Inter-reader and intra-reader variability	No	Yes
TBI vs. TB disease	Do not distinguish	Do not distinguish
Negative test	A negative test does not exclude TB infection or active TB disease	A negative test does not exclude TB infection or active TB disease
Positive predictive value [34]	QFT-GIT: 2.8 to 14.3% T-SPOT.TB: 3.3 to 10%	2.3 to 3.3%
Negative predictive value	QFT-GIT: 98.0% T-SPOT.TB: 98.3%	TST(≥ 10 mm): 100%
Immunosuppressive state	IGRA is better than TST	Low sensitivity in immunosuppressed patients
Serial testing	Conversions and spontaneous reversions	Potential boosting
Effect of BCG vaccination	Not affected by BCG	Affected by prior BCG vaccination
Window period	4 to 7 weeks	8-12 weeks
Specificity [33]	QuantiferON assay among non-BCG-vaccinated: 99% (95% CI 98 to 100%) BCG-vaccinated: 96% (95% CI 94 to 98%) T-SPOT.TB: 93% (CI 86 to 100%)	88.7% (95% CI 84.6 to 92.0) [34]
Sensitivity [33]	QuantiferON-TBGold: 78% (95% CI 73 to 82%) QFT-GIT: 70% (95% CI 63 to 78%) T-SPOT.TB: 90% (95% CI 86 to 93%)	77% (95% CI 71 to 82%)

IGRA, interferon- γ release assay; TST, tuberculin skin test; PPD, purified protein derivative; ESAT-6, early-secreted antigenic target; CFP-10, culture filtrate protein; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot assay BCG, Bacillus Calmette-Guérin; NTM, nontuberculous mycobacterium; TB, tuberculosis; TBI, tuberculosis infection; QFT-GIT, QuantiFERON Gold In-Tube; CI, confidence interval.

tion. The mitogen tube indicates patients' immune status and whether the blood handling and incubation were correctly done [49]. Results are expressed after subtracting the nil tube value from the values of the TB1 and TB2 tubes. The result is considered positive when the IFN- γ levels of the TB1 or TB2 tube minus the nil tube are ≥ 0.35 IU/mL. The result must be interpreted along with nil

and mitogen tube values. A negative test result means IFN- γ levels of less than 0.35 IU/mL in both TB antigen tubes. Indeterminate results include a nil tube value of more than > 8 IU/mL with any mitogen level and antigen tube values or a mitogen tube value of < 0.5 IU/mL with any nil and antigen tube values. Table 2 shows the interpretation strategy of QFT-Plus assay [47].

Table 2. Interpretation strategy of QFT-Plus assay [47]. Reproduced with permission from QUANTIFERON gold plus (Interferon-gamma). Available from: https://www.quantiferon.com/wp-content/uploads/2017/04/English_QFTPlus_ELISA_R04_022016.pdf.

Negative control (NIL)	Positive control (Mitogen)	TB1-NIL and/or TB2-NIL	Result
≤ 8 IU/mL	Any	≥ 0.35 and $\geq 25\%$ of the NIL value	Positive test and M.tb infection likely
≤ 8 IU/mL	≥ 0.50 IU/mL	< 0.35 or ≥ 0.35 and $< 25\%$ of the NIL value	Negative and M.tb infection NOT likely
> 8 IU/mL	Any	Any	Indeterminate
≤ 8 IU/mL	< 0.50 IU/mL	< 0.35 or ≥ 0.35 and $< 25\%$ of the NIL value	

M.tb, *Mycobacterium tuberculosis*.



Method of performing the T-SPOT.TB assay

T-SPOT.TB is an *in vitro* diagnostic test performed on the ELISpot platform. It measures the IFN- γ -producing T-cells in response to stimulation with ESAT-6 and CFP-10. Both the QFT-Plus assay and the T-SPOT.TB test will be positive, not only for *M.tb* but also for other members of the complex, including *M. bovis*, *M. africanum*, *M. microti*, *M. canetti* [50]. After a venepuncture into a heparinized tube, PBMCs are isolated, washed, counted, and normalized to create a standard cell suspension. A standard number of PBMCs (250,000 PBMCs per well) are added to a specially designed 96-well plastic microtiter plate. A total of four wells are required for each patient. Two quality control wells, a negative and a positive control, are run with each individual sample. The remaining two wells are reserved for the TB antigens. These include panel A (ESAT-6) and panel B (CFP-10) antigens. If primed T-cells are present, they will bind to TB-specific antigens and produce IFN- γ . High-affinity antibodies have already been pre-coated on the plates, allowing them to capture the released IFN- γ [50]. A 16- to 20-hour incubation in a humidified incubator at 37°C with 5% carbon dioxide is followed by the addition of a secondary enzyme-labeled antibody. [50] This will bind to the IFN- γ secreted by the T-cells and the captured anti- IFN- γ antibodies. The number of IFN- γ -producing cells is quantified as a spot number.

Blood samples to be processed with the T-SPOT.TB test must be used within 8 hours post venepuncture. Samples can be used up to 32 hours post venepuncture with the addition of the T-cell Xtend reagent prior to running the T-SPOT.TB test.

Interpretation

Results are reported as the number of IFN- γ -producing T-cells, calculated by subtracting spot-forming cells in the nil well from those of panels A and B. The positive control or mitogen tube typically has a spot count ≥ 20 spots. If the spot count in the positive control is less than 20 spots, it indicates that a subset of patients may have T-cells with poor response to phytohemagglutinin. The test result is positive if panel A-nil and/or panel B-nil results show ≥ 8 spots. Nil control value is ≤ 10 and mitogen spot count can be any [50]. A negative result is reported if (panel A-nil) and (panel B-nil) results show ≤ 4 spots. Nil control value is ≤ 10 and mitogen spot count is ≥ 20 spots. A nil control spot count > 10 spots should be reported as “invalid” or “indeterminate” and the test should be repeated. If the mitogen well shows < 20 spots and both panel A-nil and panel B-nil show ≤ 4 spots, the test should be considered invalid [50]. A borderline (equivocal) result means the highest of either “panel A-nil” or “panel B-nil” counts is 5, 6 or 7 spots. The test should be repeated. Table 3 shows the interpretative strategy of T-SPOT.TB test [51].

Table 3. Interpretative strategy of T-SPOT.TB test [50,51]. Reproduced with permission from Oxford Immunotec Limited. T-Spot.TB [Package insert]. Available from: <http://www.oxfordimmunotec.com/USpageInsert>.

Result	NIL	<i>M. tuberculosis</i> antigen panel A - NIL and/or Panel B - NIL	Mitogen	Interpretation
Positive	≤ 10 spots	≥ 8 spots	Any	<i>M. tuberculosis</i> infection likely
Negative	≤ 10 spots	≤ 4 spots	≥ 20 spots	<i>M. tuberculosis</i> infection unlikely
Borderline	≤ 10 spots	5, 6 or 7 spots	Any	Likelihood of <i>M. tuberculosis</i> infection uncertain
Indeterminate	> 10 spots ≤ 10 spots	Any ≤ 4 spots	Any < 20 spots	Likelihood of <i>M. tuberculosis</i> infection uncertain

Limitations

The IGRA test has a few limitations too. The assays require sophisticated laboratories with trained manpower and costly logistics. Furthermore, there are issues related to sample transport and storage. This is particularly relevant in developing nations. Both TST and IGRA cannot differentiate between TBI and TB disease. IGRA should not be used for active TB diagnosis in high-burden countries such as India [52]. Otherwise, there will be a significant overdiagnosis of TB due to the high prevalence of TBI in India [53]. A positive IGRA result may indicate active TB disease or TBI. A negative IGRA result cannot be used as a rule-out test [54].

False+ve interferon- γ release assay

IGRA can show false-positive in NTM infections such as *M. kansasii*, *M. szulgai*, *M. marinum*, and *M. flavescens*, as they also possess the RD-1 genes encoding CFP-10 and ESAR-10 [18,19,55]. IGRA may show false positive in *M. bovis* infection but not in the case of the BCG strain as the RD-1 was eliminated early in the process of attenuating the pathogenic strain to a nearly avirulent BCG strain [56]. Pre-analytical processing error may also cause false positive IGRA.

False-negative interferon- γ release assay

False-negative results have been identified in 8-19% of cases [57]. Elderly, underweight, HIV co-infection, extrapulmonary TB, and an increased number of human leukocyte antigen DRB1*0701 alleles were associated with false-negative IGRA results in earlier studies [57-60]. However, the limitations of these studies included small sample sizes, observational design, and being performed at a single center [57]. The Tuberculosis Network European Trials Group identified advanced age as the only risk factor for false-negative IGRA results in patients with active TB [57]. Yamasue *et al.* [61], in a systematic review, reported the following factors as contributing to false negative IGRA assay results: advanced age and low peripheral lymphocyte counts [pooled odds ratio (OR) 2.06; 95% CI 1.68-2.52 and 2.68; 95% CI, 2.00-3.61, respectively]. Wang *et al.* reported on multivariate analysis that younger age (≤ 9 years; OR=4.782; 95% CI 1.689-13.539), weight for age (z-score > 0.37 ; OR=4.256; 95% CI 1.458-12.428), and hypoproteinemia (total protein ≤ 68.4 g/L; OR=7.131; 95% CI 1.864-27.271) were risk factors for false-negative T-SPOT.TB results in childhood TB [62]. Advanced age alters the TB antigen-induced IFN- γ production, whereas a low peripheral blood lymphocyte count determines the IFN- γ production in QFT but not T-SPOT. This is because the T-SPOT assay needs a certain minimum amount of PBMCs. Compartmentalization of T-cells within the site of pathology may explain the false-negative result.



Technical-operational variability may also cause a false-negative assay. False-negative QFT test results are more common in HIV-infected individuals with lower CD4 counts [63].

Indeterminate results

Indeterminate results indicate either high IFN- γ levels (>8 IU/mL) in the nil tube or low IFN- γ levels (<0.5 IU/mL) in the mitogen tube. The indeterminate result with T-SPOT.TB is defined by a nil control spot count of >10 spots. Zhou *et al.*, in a systematic review and meta-analysis, reported a pooled indeterminate rate of 3.9% (95% CI 3.5–4.2%) for IGRAs in the detection of TBI [64]. They also found a similar indeterminate rate for QuantiFERON®-TB and T-Spot.TB; however, QuantiFERON-Plus had a lower indeterminate rate than T-Spot.TB. Diel *et al.* reported the pooled rate of indeterminate results of the T-Spot.TB as 3.8% (95% CI, 0.035–0.042) and 6.1% (95% CI 0.052–0.071), among immunocompetent and immunosuppressed patients, respectively [65]. Important risk factors for indeterminate IGRA results are immunosuppression and children, especially those younger than 5 years. The indeterminate rate in the immunocompromised population was significantly higher than that in healthy controls (OR=3.51, 95% CI 2.11–5.82).

A lower CD4+ cell count in HIV-positive patients further increased the rate of indeterminate values. Compared to adults, children's pooled indeterminate rates (OR=2.56, 95% CI 1.79–3.57) were significantly higher. These rates increased as the children's age dropped [64]. The majority of indeterminate results are due to failed positive controls (94.6%, 95% CI 89.6–98.0%) [65]. This indicates dysfunctional T lymphocytes or technical errors. Failed nil tubes may be due to the presence of heterophilic antibodies (e.g., human anti-mouse) or spontaneous IFN- γ secretion during an infection or following vaccination [66]. Sun *et al.* [67], in a multivariate analysis, reported that being male (OR=1.882, 95% CI 1.222–2.899), Behcet's disease (OR=7.764, 95% CI 1.714–35.167), heavy use of corticosteroids within a month (OR=0.357, 95% CI 0.138–0.921, for the >1000 mg group), and hypoalbuminemia (OR=0.385, 95% CI 0.241–0.615) are significantly associated with high nil responses. Another cause of an indeterminate QuantiFERON-Plus result is severely ill COVID-19 patients. Ward *et al.* found a 6-fold reduction of IFN- γ levels compared to control patients in severely ill COVID-19 patients [68]. More than 60% of these severely ill COVID-19 patients showed poor mitogen responses. Low mitogen levels are associated with age <10 years [adjusted OR (aOR) 3.7, 95% CI 2.4–5.9], females (aOR 1.4, 95% CI 1.1–1.8), Asians (aOR 2.1, 95% CI 1.3–3.4), and the US-born (aOR 1.9, 95% CI 1.4–2.6). A high nil results are more frequent among Hispanics (aOR 1.7, 95% CI 1.0–2.8) [69]. The indeterminate IGRA result has been reported in 27.1% of the HIV-infected patients (95% CI: 17.8–38.1%), and the risk increased in patients with advanced immunosuppression (CD4+ T-cell count <200 cells/mm³) [70]. The T-SPOT.TB is associated with significantly less frequent indeterminate results than QFT-G (3% vs. 11%, $p<0.0001$) [71]. Patients on immunosuppressive therapy and peripheral blood lymphocytopenia have the highest rate of indeterminate results. Therefore, T-SPOT.TB is not only more sensitive in immunocompromised persons, but it also produces significantly fewer indeterminate results compared to the QFT-G assay. Therefore, in an HIV-infected patient with lower CD4+ lymphocyte counts, T-SPOT.TB would be a reasonable choice [72]. IGRA tests are also preferred in subjects vaccinated with BCG and in children >5 years [73]. The increased sensitivity of the T-SPOT.TB

test should be considered with the caveat that T-SPOT.TB test may detect a transient immune response to TBI [74]. Table 4 shows the difference between QuantiFERON-TB Plus assay and the T-SPOT.TB (T-SPOT) assay.

Variability of interferon- γ release assay results

Another issue with the IGRA assay is the poor reproducibility on serial testing, particularly among healthcare workers (HCWs). On serial testing, IGRA shows a higher rate of reversions and conversions. Conversions occur in the apparent absence of TB exposure, whereas reversions occur in the absence of treatment [75]. Joshi *et al.* retrospectively evaluated the long-term reproducibility, conversions, reversions, and predictive value data of QFT-GIT among 2303 HCWs in the United States [76]. They found reversions and conversions in 45% and 3.2%, respectively. They performed a third QFT-GIT among 41 of 71 converters, and 90% of them had reversions. This indicates poor reproducibility of this test. Therefore, QFT-GIT is not suitable for serial testing.

Pai *et al.* considered true conversions for individuals with fluctuating QFT-GIT results close to the cut-off value of 0.35 IU/mL [77]. These conversions and reversions tend to occur more frequently when the initial result is close to the cut-off (0.35 IU/mL). Thanassi *et al.* conducted serial QuantiFERON-GIT tests in US HCWs, a low-risk population [78]. They reported reversions in 52.2% of initial positive cases, and they occurred most likely when the TB antigen-nil was 1.11 IU/mL. Dorman *et al.*, in the same low-risk cohort, reported reversions of QFT-GIT and T-SPOT.TB in 76.4% and 77.1%, respectively when retested after 6 months [79]. This indicates a false positive IGRA in a low-risk population, which can result in unnecessary chest x-rays, unwarranted anxiety and worry, and often unnecessary treatment and sometimes side effects [80]. van Zyl-Smit *et al.* found within-subject variability of IGRA of 16 to 80% [81]. IGRA variability can occur due to manufacturing sources, pre-analytical sources, analytical sources, and immunological sources [82]. The pre-analytical factors that may explain IGRA variability include blood volume, sample agitation, temperature during transport, delay in incubation, duration of incubation, plasma separation delay, and plasma/PBMC storage [83]. The variability also includes intra-laboratory and inter-laboratory variability [84].

One important pre-analytical delay is the incubation delay. Although the manufacturer of the QuantiFERON assay recommends a 0 to 16-hour range of delay before incubation, Doberne *et al.* showed that 6- and 12-hour delays caused reversion rates of 19% and 22%, respectively when compared to immediate incubation [85]. Incubation delays may also cause a rise in indeterminate results [82]. Other pre-analytical factors include methods of shaking, variation in blood volume, and incubation duration [82]. Tagmouti *et al.*, in a systematic review, found that under ideal conditions (*i.e.*, repeat testing of an aliquot of the same sample), the QuantiFERON IFN- γ result could vary by ± 0.47 IU/mL (coefficient of variation, 13%) and ± 0.26 IU/mL (30%) for individuals with an initial IFN- γ response in the borderline range (0.25–0.80 IU/mL) [86]. They identified blood volume inoculated into QuantiFERON tubes and pre-analytic delay as key sources of variability. An immunological source of variability includes immune-boosting phenomena as reported by van Zyl-Smit *et al.* [81]. They showed a TST-induced “boosting” of IGRA responses, which occurred after 3 days and may persist for several months. IGRA should be performed within 72 hours of PPD placement [87]. The Canadian guideline on IGRA for LTBI stated that if



Table 4. Difference between QuantiFERON-TB plus assay T-SPOT.TB assay.

	QuantiFERON-TB assay	T-SPOT.TB assay
Setting of the test	<i>In vitro</i> diagnostic Indirect test to assess adaptive immunity for TB	<i>In vitro</i> diagnostic Indirect test to assess adaptive immunity for TB
Basic feature	Enzyme-linked immunosorbent assay-based assay It uses whole-blood Used nil, positive control, and one antigen tube in QFT-GIT QFT-Plus assay uses both long and short peptides of ESAT-6 and CFP-10 in TB1 and TB2 tube	Enzyme-linked immunospot assay It uses peripheral blood mononuclear cells Used Nil, positive control, and 2 antigen tubes (TB1 and TB2)
Antigens used	QuantiFERON-TB: PPD QuantiFERON-TB Gold: ESAT-6 and CFP-10 QFT-GIT: ESAT-6, CFP-10, and TB7.7 QFT-plus: ESAT-6 and CFP-10	ESAT-6 and CFP-10
Principle of the test	Detect IFN- γ produced by <i>Mycobacterium tuberculosis</i> primed T lymphocytes following exposure to TB antigens	Detect IFN- γ produced by <i>Mycobacterium tuberculosis</i> primed T lymphocytes following exposure to TB antigens
Infection and disease	Unable to differentiate infection from active TB disease	Unable to differentiate infection from active TB disease
Effect on T-lymphocytes	QFT-plus stimulates both CD4+ and CD8+ T-lymphocytes	Stimulates both CD4+ and CD8+ T-lymphocytes
Boosting effect on repeat test	No	No
Need for return visit	No	No
Output	Measures serum concentration of IFN- γ produced by T cells on contact with ESAT-6 and CFP-10	Measures spots produced by IFN- γ secreted by T cells on contact with ESAT-6 and CFP-10
Number of venipuncture	Single blood draw	Single blood draw
Incubation time	16–24 h [48]	16–20 h [48]
Interpretation	Positive (≥ 0.35 IU/mL) Negative (< 0.35 IU/mL) Indeterminate: low mitogen and/or high nil	Positive (8 spots) Negative (4 spots) Indeterminate/invalid: low mitogen and/or high nilborderline (5 to 7 spots)
Sensitivity	QuantiFERON-TB Gold: 78% (95% CI 73 to 82%) QFT-GIT: 70% (95% CI 63 to 78%) [33] QFT-Plus: 0.91 (95% CI 0.84 to 0.96) [36]	90% (95% CI 86% to 93%) [33]
HIV-infected individuals [49]	Sensitivity: QFT-GIT 61% (95% CI 41 to 75%)	Sensitivity: 72% (95% CI 62-81%)
Specificity	Non-BCG-vaccinated: 99% (95% CI 98 to 100%) BCG-vaccinated: 96% (95% CI 94 to 98%) [33] 0.95 (95% CI 0.93 to 0.97) [36]	93% (CI, 86% to 100%) [33]
Effect of BCG	No effect	No effect
PPV [34]	QFT-GIT 2.8 to 14.3%	3.3 to 10%
NPV [34]	QFT-GIT 99.8% (95% CI 99.4-100)	97.8% (95% CI 94.5-99.4%)
Indeterminate results	QFT-G: 2% [69] Immunosuppressed: 11% [71]	Immunocompetent: 3.8% (95% CI 0.035-0.042) [65] Immunosuppressed: 6.1% (95% CI 0.052-0.071) [65]

TB, tuberculosis; QFT-GIT, QuantiFERON Gold In-Tube; QFT-Plus, QuantiFERON-TB Gold Plus; ESAT-6, early-secreted antigenic target; CFP-10, culture filtrate protein; TB1, TB antigen tube 1; TB2, TB antigen tube 2; PPD, purified protein derivative; IFN- γ , interferon- γ ; BCG, Bacillus Calmette-Guérin.

both TST and IGRA tests are performed, the blood sample for IGRA should be obtained on the same day as the TST [88]. If a TST was administered, the Wisconsin TB Program recommends IGRA testing at least 90 days after a TST to avoid potential boosting [89].

Another issue is the discordance between IGRA and TST results, especially in children. The most common discordant pattern in the low-TB endemic setting is TST-positive and IGRA-negative. It may be due to false-negative IGRA or false-positive TST. IGRA can be falsely negative during the window period. Usually, it takes 4 to 6 weeks after exposure for IGRA conversion. However, sometimes, 14 to 22 weeks may elapse after exposure for IGRA conversion [90]. Pregnancy is an immunosuppressive state. Weinberg *et al.* found that from entry to delivery, 24% of 284 QFT-GIT-positive women reverted to a negative or indeterminate state [91]. A decreased IFN- γ production in response to TB antigen and/or mitogen during pregnancy

may explain the loss of QFT-positivity during pregnancy. Unlike TST, IGRAs are not affected by prior BCG vaccination. Therefore, in countries where adult BCG vaccination has been adopted, IGRA should be done instead of TST. Intravesicular BCG administration for bladder cancer also affects the result of TST but not IGRA. Silverman *et al.* showed that intravesicular BCG administration predicted a positive TST ($p < 0.001$) response but not a positive IGRA ($p = 0.35$) [92].

Novel skin tests

Newer M.tb antigen-based skin tests (TBSTs) using the M.tb-specific antigens (ESAT-6 and CFP-10) seem very promising for the diagnosis of TBI. The WHO assessed the outcomes of these three



TBSTs: Cy-Tb (Serum Institute of India, India); C-TST (formerly known as the ESAT-6-CFP-10 test, Anhui Zhifei Longcom, China); and Diaskintest (Generium, Russian Federation). All three TBSTs use intradermal injection of antigen and are read after 48–72 hours as induration, similar to the method suggested for the Mantoux test. The sensitivity of the new TBST was similar to TST and IGRA, and specificity was similar to that of IGRA and better than that of TST, particularly in populations with prior BCG vaccination history [93]. These tests combine the operational advantages of the TST and the specificity of IGRAs for the diagnosis of TBI [94]. Similar to TST, all these three new skin tests require two clinic visits.

Cy-Tb

It was formerly known as the C-Tb test. It consists of a 1:1 mixture of two RD-1 antigens as a recombinant dimer (rdESAT-6 and CFP-10). These antigens are produced by a recombinant method in *Lactococcus lactis* by the SSI, Denmark [95]. The dose is applied intradermally (0.1 mL) and contains 0.05 µg of rdESAT-6 and 0.05 µg of rCFP-10 [93]. The cut-off of induration is 5 mm. The storage is at 2–80°C. [96] The Serum Institute of India, in a partnership with SSI, manufactures and distributes this test. Ruhwald *et al.* [94], in a double-blind, randomized, controlled phase 3 trial, evaluated the efficacy and safety of the C-Tb skin test in the diagnosis of TBI in Spain. They also compared the result with QFT-GIT and TST tests. Unlike TST, there was no impact of BCG vaccination on C-Tb results. The Cy-Tb positivity was 43% in close contacts. Furthermore, there was strong concordance between Cy-Tb and QFT-GIT results (94%). They reported a similar safety profile of C-Tb to that of the TST. The specificity of the C-Tb test was 99.3% (95% CI 96–100%) [95]. The sensitivity using a cut point of 5 mm was 73.9 (95% CI 67.8–79.3) [96,97].

Diaskintest

Diaskintest is produced and marketed by Generium Pharmaceutical, Russia. The recombinant protein is produced by genetically modified *Escherichia coli* BL21 and subsequently diluted with sterile isotonic phosphate buffer solution along with a preservative (phenol) [93]. The dose is applied intradermally (0.1 mL) and contains 0.2 µg of ESAT-6 and CFP-10 recombinant protein and auxiliary ingredients. The result is read after 48 to 72 hours. The presence of induration or a papule of any size is considered a positive reaction. The storage is at 2–80°C [96]. Starshinova *et al.* [98], in a meta-analysis, included results of 3,777,083 patients tested with Diaskintest (83.0%). They reported the overall diagnostic sensitivity and specificity of 86.0% and 98.0%, respectively, with an accuracy of 95.1% in the total studied population.

C-TST

Formerly called the ESAT-6-CFP-10 test (Anhui Zhifei Longcom, China), the C-TST test uses the recombinant fusion protein ESAT-6-CFP-10 (EC) produced in genetically modified *Escherichia coli*. The test dose is applied intradermally (0.1 mL) and contains 5 units (U) of recombinant M.tb fusion protein, phosphate-buffered saline (PBS, 1.0 mmol/L), phenol (3%) and Tween 80 (0.0005%) [99]. An average diameter of induration or erythema is measured by summing the transverse and longitudinal diameters and the whole divided by 2. Zhang *et al.* reported that an induration or erythema diameter not less than 5 mm was useful in the diagnosis of TBI [99]. The manifestations that are considered strong positive reactions include blister, skin necrosis, and lymphadenopathy. In a

phase 2b trial, Xu *et al.* found sensitivity and specificity of 87.5% (95% CI 77.8–97.2) and 98.9% (95% CI 96.0–99.9), which were similar to those of T-SPOT.TB test, 86.5% (95% CI, 79.5–93.4) and 96.1% (95% CI, 93.5–97.8), respectively [100].

Newer interferon-γ release assay tests

The LIAISON QFT-Plus assay is a chemiluminescence immunoassay analyzer that measures IFN-γ using the fully automated LIAISON® XL analyzer [101]. The manufacturer is the DiaSorin S.p.A., Italy. This test is called QFT-Plus chemiluminescent immunoassay (CLIA) or LIAISON QFT-Plus assay. Fernández-Huerta *et al.* compared the QFT-Plus assay on Liaison XL and ELISA platforms in Barcelona, Spain [102]. They observed substantial agreement ($\kappa=0.872$) and correlation ($r>0.950$) between the two assays. The QFT-Plus assay on the Liaison XL platform generated significantly higher levels of IFN-γ IU/mL than the ELISA ($p=0.004$ for the TB1 tube and $p=0.010$ for the TB2 tube). The higher IFN-γ levels also allowed interpretable results, which were indeterminate on the ELISA platform. Therefore, the LIAISON QFT-Plus assay is more sensitive compared to the ELISA-based QFT-Plus assay. It measures IFN-γ produced by both CD4+ and CD8+ lymphocytes. Bisognin *et al.* similarly reported substantial agreement between the two (75.4%) [103]. The Italian Clinical Microbiologist Association defined borderline results as TB1 and/or TB2 values within the range of 0.20 to 0.70 IFN-γ IU/mL and recommended retesting. The chemiluminescence-based assay significantly increased the IFN-γ levels from 0.29 to 0.59 IU/mL in TB1 and 0.32 to 0.60 IU/mL in TB2, respectively, although it remained in the borderline range. The receiver operating characteristic (ROC) analysis suggested the optimal cut-off value of 0.45 IU/mL for TB1 and 0.46 IU/mL for TB2. Being automatic in nature, it has reduced assay time. The incubation period is 16 to 24 hours with an assay time of 45 minutes. It can perform up to 25 tests per hour [104]. Altawallbeh *et al.* reported excellent agreement of 97.8% agreement between the LIAISON and QFT-Plus assays among 185 potential TB samples with Cohen's κ value of 0.88 [105]. Buron *et al.* found higher IFN-γ measurements across the range of values with the CLIA than with the ELISA [106]. CLIA was positive in 42.7% of borderline-negative results with ELISA. Therefore, there is risk of a false-positive result with the Liaison CLIA for QFT-Plus in low-incidence settings. Confirming borderline positives with ELISA is a viable strategy to mitigate false-positive CLIA results.

QIArearch® QuantiFERON-TB

The QIArearch QuantiFERON-TB (QIArearch) (QIAGEN, Carnegie, Australia) is a semi-automated assay that incorporates digital fluorescence lateral flow nanoparticle technology to capture the IFN-γ level released by both CD4 and CD8 T-cells in plasma [107]. It uses only the TB2 tube of QTF plus assay and does not use mitogen or nil tubes. It measures IFN-γ in plasma via its unique digital detection cartridge known as eStick attached to the power source (8-port eHub portable platform). The assay has the capability to perform 24 tests per eHub per hour. The test result is available on the eHub display in 3–20 minutes. Stieber *et al.* [108], in a feasibility study, reported overall agreement between QIArearch-QFT and QFT-Plus assays of 95.6% (two-sided 95% CI 91.8–98), with a positive percentage agreement (*i.e.*, sensitivity) of 100% (95% CI 94.7–100) and a negative percentage agreement (*i.e.*, specificity) of 95.6% (95% CI 90.6–98.4). The main advantage of QIArearch-QFT is the



requirement of less laboratory infrastructure and resources. It is also portable and requires only 1 mL of blood [109]. Furthermore, it has a rapid turnaround time. In one study, the sensitivity of QIArearch QFT for diagnosing TBI was 93.7% (two-sided 95% CI 82.2-98.7%) and 95.1% (two-sided 95% CI 88-98.7%), respectively for the untreated and treated groups, whereas the specificity was 97.7% (two-sided 95% CI 94.2-99.4%). The overall percentage agreement with QFT-Plus was 95.7% (two-sided 95% CI 92.8-97.7%) with a Cohen's κ of 0.96 [107]. In a systematic review and meta-analysis, the pooled sensitivity, specificity, PPV, and NPV of QIArearch QFT for diagnosing TBI were 99% (95% CI 95-100%), 94% (95% CI 85-97%), 88% (95% CI 70-98%), and 100% (95% CI 99-100%), respectively [110].

VIDAS TB-IGRA (bioMérieux, France)

VIDAS® TB-IGRA is a fully automated in vitro diagnostic for TBI. It reduces the chance of human error as it is fully motivated. It requires incubation at 37°C for 16 hours with a turnaround time of 17 hours [111]. It uses three tubes: nil, antigen, and mitogen tubes. The positive result and negative results are defined by antigen-Nil IFN- γ concentrations ≥ 0.35 IU/mL and ≤ 0.35 IU/mL, respectively. An indeterminate result is defined by mitogen-nil IFN- γ concentrations of < 1.1 IU/mL or by a nil IFN- γ concentration of ≥ 6.4 IU/mL. In one multicentric study, VIDAS® TB-IGRA produced a smaller number of indeterminate results compared to QFT-Plus (1/107 vs. 23/107) in patients with TB disease [111].

LIOFeron®TB/LTBI IGRA (LIONEX Diagnostics & Therapeutics, Germany)

LIOFeron®TB/LTBI IGRA assay includes two components: the human blood stimulation tubes and the IFN- γ ELISA. This test is a CLIA. There are four blood tubes: positive control, negative control, TB A, and TB B [112]. ESAT-6, CFP-10, and TB7.7 antigens are present in TB A tube, whereas alanine dehydrogenase (Ala-DH) is present in TB B tube [49]. This test measures IFN- γ produced by both CD4+ and CD8+ lymphocytes. Ala-DH is the proprietary antigen of LIONEX. It has several advantages. It is absent in BCG [113]. It contains the MHC class I-restricted T CD8+ lymphocytes epitopes [114]. This antigen is also involved in adaptation to the anaerobic dormant stage of latent TBI [115]. There is less manual handling as the test measures IFN- γ concentrations automatically [104]. The incubation time is 16-24 hours at 37°C with an assay time of 2.5 hours [104,112]. The positive result is defined by antigen-nil IFN- γ concentrations ≥ 0.35 IU/mL and $\geq 25\%$ of the negative control. The negative result is defined by antigen-Nil IFN- γ concentrations < 0.35 IU/mL or $< 25\%$ of the negative control and the positive control is ≥ 0.5 IU/mL. An indeterminate result includes negative control IFN- γ concentrations of > 8 IU/mL or antigen tube-nil IFN- γ concentrations of < 0.35 IU/mL or $< 25\%$ of the negative control with a mitogen control of < 0.5 IU/mL. Bella *et al.* reported sensitivity and specificity of LIOFeron®TB/LTBI IGRA assay for TBI diagnosis as 90% and 98%, respectively, whereas those of the QFT-Plus assay were 85% and 94%, respectively [116]. Therefore, it has higher sensitivity than the QFT-Plus assay.

Ichroma™ IGRA-TB

The Ichroma™ IGRA-TB is produced and marketed by Boditech Med Inc., Chuncheon, Republic of Korea. It is a fully automated point-of-care device with three tubes, namely, nil, mitogen, and TB antigen tubes. It uses a fluorescent lateral flow immunoassay

to measure IFN- γ concentration [117]. The antigens used are ESAT-6 and CFP-10. The incubation time is 16 to 24 hours and results are obtained in 15 minutes [117]. The ichroma™ IGRA-TB showed an acceptable performance compared to the QFT-Plus assay. However, there was a higher frequency of indeterminate results (2.2%) compared to the QFT-Plus assay [118]. The total agreement rate and κ value between the ichroma™ IGRA-TB and QFT-Plus were 95.2% and 0.70 (strong agreement), respectively [118].

STANDARD F TB-Feron FIA

The STANDARD F TB-Feron FIA (SD Biosensor, Republic of Korea) is a fluorescent lateral flow immunoassay [119]. It contains three tubes, with the TB antigen tube containing ESAT-6, CFP-10, and TB7.7 similar to QFT-GIT [120]. However, there are certain differences between the STANDARD F TB-Feron FIA and QFT-GIT. The TB antigens in the former contain recombinant whole proteins of ESAT-6, while the latter contains TB-specific synthetic peptide antigens. [104] Whole proteins break down into several small peptides with various epitopes and the advantage of using them is their enhanced sensitivity. Therefore, it can stimulate T-cells vigorously and has thereby increased IFN- γ levels [121]. The incubation time of the STANDARD F TB-Feron FIA is 16 to 24 hours at 37°C and the result is available in 15 minutes [49]. Yoo *et al.* reported an agreement rate between STANDARD F TB-Feron FIA and QFT-Plus of 92.0% with a Cohen's κ value of 0.77 (95% CI 0.68-0.87) [122].

Erythra TB-KIT (Erythra Inc., Stanford, CA, USA)

This test uses PPS as antigen stimulation for whole blood. The technology is based on a lateral flow chromatography assay. It does not require any incubation. The assay time is 20 minutes with a throughput of one sample per run [96].

AdvanSure 13

It is a chemiluminescence-based assay manufactured by LG Chem, Seoul, Korea. It measures IFN- γ response to ESAT-6, CFP-10, and TB7.7 antigens quantitatively and automatically. [96] It uses small assay volumes of 50 microliters of incubated plasma from each of the three tubes for analysis [123]. The incubation time is 16 to 24 hours and the analysis time post-incubation is 15 minutes [96,124]. Magnetic-coated anti-human IFN- γ antibodies were used for the detection. The resultant anti-human IFN- γ antibody-IFN- γ complex produces a chemiluminescent signal, which is measured as a relative light unit (RLU). It correlates with the amount of IFN- γ [123]. Kim *et al.* evaluated the performance of AdvanSure I3 and QFT-GIT assays using 341 blood samples from HCWs and patients and reported 99.1% overall agreement ($\kappa=0.98$) between the two assays [123].

IP-10 IGRA

Serum levels of cytokine such as IFN- γ , induced protein 10 (IP-10) in patients with TB [96]. IP-10 is secreted by antigen-presenting cells upon stimulation by IFN- γ and has a higher expression compared to IFN- γ . The IP-10 IGRA is a quantitative lateral flow assay developed by R-Biopharm (Pfungstadt, Germany). The incubation time is 16 to 24 hours and the analysis time post-incubation is 20 minutes. Ortiz-Brizuela *et al.* [125], in a systematic review and meta-analysis, evaluated the diagnostic performance of new commercial IGRA for TBI. They included a total of 87 studies (QFT-Plus 44, 10 for Standard E TB-Feron, 4 for QFT-Plus CLIA, 3 for QIArearch, 26 for TB-IGRA, and 1 T-SPOT.TB). Furthermore, as



compared to the QFT-GIT, the sensitivity of QFT-Plus was 0.1 percentage points lower [95% CI -2.8 - 2.6; certainty of evidence (CoE): moderate], and its specificity was 0.9 percentage points lower (95% CI -1.0 - 0.9; CoE: moderate). However, as compared to QFT-GIT, the sensitivity and specificity of TB-IGRA were 3.0 percentage points higher (95% CI -0.2 - 6.2; CoE: very low) and 2.6 percentage points lower (95% CI -4.2 - 1.0; CoE: low), respectively. They also found an excellent agreement between the QFT-Plus CLIA, QIArearch, and QFT-Plus.

Conclusions

Diagnosis of TBI and TB-preventive therapy are important steps in achieving the End TB strategy. There is no gold standard for the diagnosis of TBI. IFN- γ -based assays are a reliable alternative to the old TST for diagnosing TBI. Both tuberculin and IGRA measure the adaptive immune response to M.tb antigens and both tests do not differentiate between TBI and TB disease.

Currently available IGRA assays include the QFT-G in a tube, QuantiFERON-plus, and the ELISpot assay. The IGRA assay is more specific compared to the tuberculin test, as this assay utilizes the RD-1 antigens such as ESAT-6 and CFP-10. These antigens are not shared by the BCG strain and the majority of non-tubercular mycobacterial infections. However, both TST and IGRA have low PPV of progression into TB disease. The ELISpot assay is more sensitive in immunocompromised populations. The QFT-Plus assay has comparable sensitivity and specificity to the QFT-G in-tube test. IGRA should be advised in BCG-vaccinated individuals. IGRA testing is not ideal for serial testing, particularly in low-risk settings. There are many newer skin and IGRA-based tests. An ideal test should fulfill WHO criteria of TPP for a test in order to predict progression from TBI to active disease. The newer TBSTs have the advantage of good specificity as they use the M.tb-specific antigens, such as ESAT-6 and CFP-10. In addition, these tests can be used at the peripheral level, unlike the IGRA tests. The newer skin-based test is also cost-effective compared to the conventional IGRA test, which needs sophisticated laboratory infrastructure.

Moreover, in resource-poor countries, shifting of IGRA samples is often difficult. This is particularly advantageous for low and middle-income countries. The Cy-TB test has already been rolled out in the national TB elimination program by the government of India. There is heterogeneity in the screening approach to patients harboring TBI, which depends on various factors such as cost-effectiveness, availability, requirement of specialized training, and, last but not least, requirement of sophisticated laboratory infrastructure. The implementation of TB preventive therapy faces several gaps worldwide. Mitigation of these gaps, including gaps in testing, will be a crucial step in achieving the end TB target.

In the future, we need newer assays for diagnosing TBI that will not only meet WHO criteria of TPP, but also possess the capability to differentiate TBI from TB disease. It should be suitable for point-of-care applicability. It should possess high sensitivity, specificity, PPV, cost-effectiveness, stability in different climates, and not require a stringent storage facility.

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