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MINIREVIEW

Interferon Gamma Release Assays for Latent Tuberculosis: What Are the Sources of Variability?

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Interferon gamma release assays (IGRAs) are blood-based tests intended for diagnosis of latent tuberculosis infection (LTBI). IGRAs offer logistical advantages and are supposed to offer improved specificity over the tuberculin skin test (TST). However, recent serial testing studies of low-risk individuals have revealed higher false conversion rates with IGRAs than with TST. Reproducibility studies have identified various sources of variability that contribute to nonreproducible results. Sources of variability can be broadly classified as preanalytical, analytical, postanalytical, manufacturing, and immunological. In this minireview, we summarize known sources of variability and their impact on IGRA results. We also provide recommendations on how to minimize sources of IGRA variability.

Interferon gamma (IFN-γ) release assays (IGRAs) are laboratory alternatives to the tuberculin skin test (TST) for diagnosis of latent tuberculosis infection (LTBI) (1). IGRAs are *ex vivo* assays that measure T-cell response after overnight stimulation with antigens that are relatively specific for *Mycobacterium tuberculosis*. IGRAs are increasingly replacing the TST for annual screening of U.S. health care workers and are also utilized in student/employee health and public health programs and in screening of patients prior to immunosuppression (1). In addition, the IGRA conversion rate is now being used as a measure of vaccine efficacy in TB vaccine trials (2).

IGRAs are supposed to offer improved specificity over the TST (1). By and large, this advantage holds true in populations that receive *Mycobacterium bovis* BCG vaccination after infancy (1 year of age) or receive multiple doses; the specificity of TST is compromised in such settings (1). However, in practice, IGRAs have proved less specific in low-risk North American health care workers and college students (1). Furthermore, several studies have raised concerns over high rates of IGRA reversions (1). Given the growing use of IGRAs, it is imperative that we identify the underlying sources of variability and understand their impact on IGRA accuracy.

The two most widely used IGRAs include the QuantiFERON-TB Gold In-Tube (QFT-GIT) assay (Cellestis/Qiagen, Carnegie, Australia) and the T-SPOT.TB (T-SPOT) assay (Oxford Immunotec, Abingdon, United Kingdom). Although a new, four-tube version of QFT (called QFT-Plus) has been launched by Qiagen, this version is currently not available in the United States. The QFT-GIT assay, the FDA-approved version, is an enzyme-linked immunosorbent assay (ELISA)-based, whole-blood test that uses peptides from the two RD1 antigens (ESAT-6 and CFP-10) as well as peptides from one additional antigen (TB7.7 [Rv2654c]) in an in-tube format. The QFT-GIT assay consists of three tubes: the negative-control (nil) tube that measures background IFN-γ response, the antigen tube that measures antigen-specific response, and the positive-control (mitogen) tube that measures nonspecific T-cell response. The qualitative result (negative, positive, or indeterminate) is interpreted from quantification of IFN-γ in international units (IU) per milliliter. An individual is considered positive for *M. tuberculosis* infection if the TB response (IFN-γ response to TB antigens minus the background IFN-γ response) is above the test cutoff value.

The T-SPOT is an enzyme-linked immunospot (ELISPOT) assay performed on separated and counted peripheral blood mononuclear cells (PBMC) after incubation with ESAT-6 and CFP-10 peptides in separate wells. The T-SPOT assay consists of four wells: the negative-control (nil) well that measures background IFN-γ-producing T cells (spot-forming cells [SFC]), the two antigen wells that measure *M. tuberculosis*-specific SFC to ESAT-6 and CFP-10, and the positive-control (mitogen) well that measures nonspecific SFC. The qualitative result (negative, positive, or indeterminate) is interpreted from the number of SFC. An individual is considered positive for *M. tuberculosis* infection if the spot counts in the TB antigen wells exceed a specific threshold after subtracting the number of spots in the negative-control well. Oxford Immunotec offers T-SPOT testing in the United States via its own national reference laboratory called Oxford Diagnostic Laboratories (Memphis, TN).

Given the multiple steps involved in IGRAs, error can be introduced at any point during testing. In addition, because IGRAs are functional assays, they are susceptible to modulation by different factors. In recent years, studies have identified and characterized various sources of variability. Most of these studies have investigated variability in the QFT-GIT assay because it is more widely used for on-site testing than the T-SPOT assay. In a previous analysis (1), we broadly classified the sources of variability as preanalytical, analytical, manufacturing, and immunological (Fig. 1). In the ensuing sections, we update our previous review and describe

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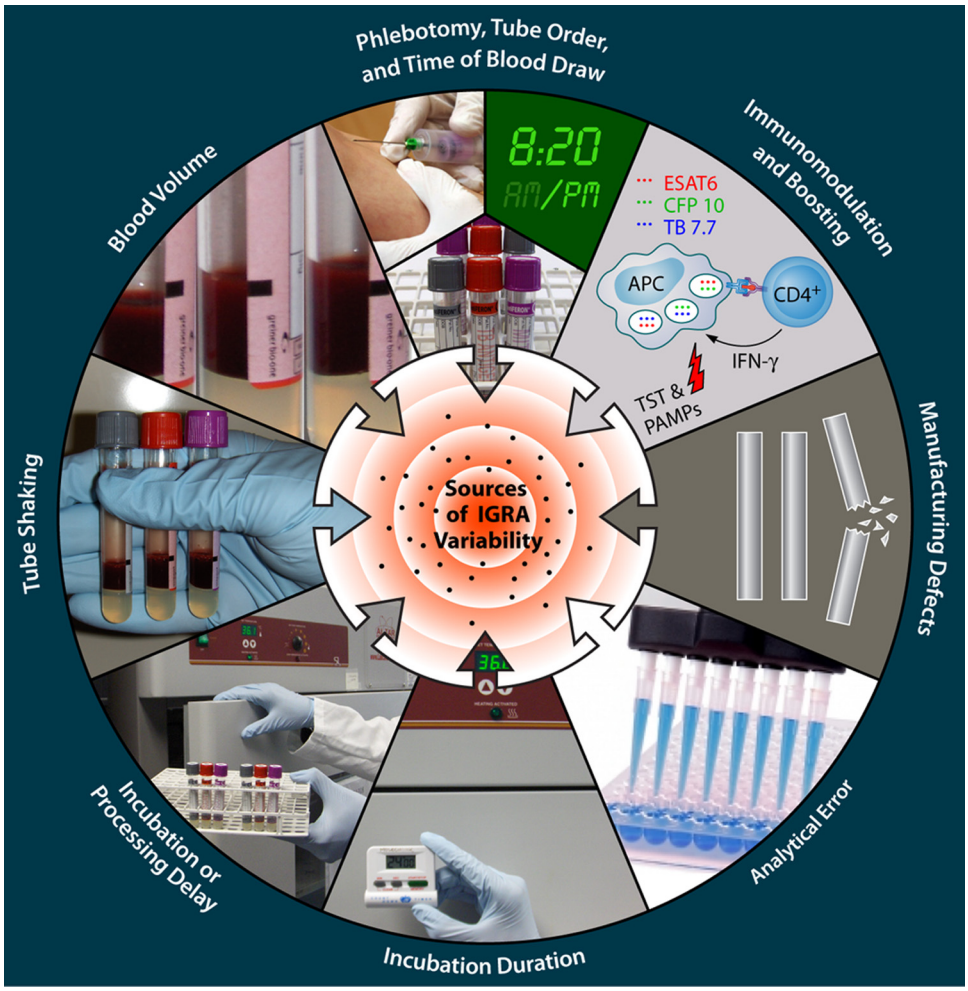

FIG 1 Major sources of variability in IGRA results. (Republished from reference 1).

each source of variability and the potential impact it may have on results (Table 1).

SOURCES OF VARIABILITY

Preanalytical sources of variability. Preanalytical sources of variability are numerous and represent some of the more important sources of IGRA variability (3). The time of blood collection might make a difference. Mazurek et al. reported significantly higher TB response values with the QFT-GIT assay when blood was collected in the evening compared to the morning (4). Phlebotomy is the first window of opportunity for introduction of predictable variability. Inadequate disinfection of the skin and the rubber septum of blood collection tubes may introduce microbial contaminants into the blood sample. The immunomodulatory effect of microbes is discussed below under immunological sources of variability. Blood collection in the correct order of QFT-GIT tubes (starting with the nil tube and ending with the mitogen tube) might matter. Contamination of the antigen tube with mitogen can cause a false-positive result, and contamination of the nil tube with mitogen can cause a false-negative result.

Next, the volume of blood drawn into the QFT-GIT antigen tube can alter results. The blood volume drawn into the Vacutainer tubes can range from 0.8 to 1.2 ml according to the package insert and a published study (5). Gaur et al. investigated the impact of 0.8, 1.0, and 1.2 ml blood added to the antigen tube and showed that blood volume inversely correlates with TB response in infected individuals and results in false-negative results in some individuals (5). Last, vigorous shaking of QFT-GIT tubes can nonspecifically increase IFN-γ response in the nil tube and antigen tube and lead to either a false-positive or false-negative result depending on which tube is shaken excessively (5).

Among the potential sources of variability shown in Fig. 1, delay in processing between the collection of blood and incubation of cells is one of the more important variables (3). According to the package inserts, incubation of the QFT-GIT tubes can be delayed for up to 16 h, and processing of a blood sample for the T-SPOT assay can be delayed for up to 8 h. However, studies using ELISPOT and noncommercial whole-blood assays have shown a processing delay of 1 to 4 h before stimulation with *M. tuberculosis* antigens results in a lower number of IFN-γ-producing T cells (6). Doberne et al. investigated the impact of incubation delay on QFT-GIT and showed a significant decline in TB response when incubation was delayed by 6 and 12 h, resulting in 19% (5/26) and 22% (5/23) false-negative rates, respectively (7). Incubation delay has also been shown to increase the indeterminate rate due to

TABLE 1 Potential sources of variability in IGRAs and their impact on assay response[a]

Potential source of variability[b]	Impact[c] of potential source of variability on the response of the following assay: QFT-GIT	T-SPOT
Manufacturing		
Between-lot variability	↑ ↓	?
Preanalytical		
Time of blood draw (a.m. vs p.m.)	↑ p.m.	?
Skin disinfection	↑ ↓	?
Traumatic blood draw	?	?
Blood vol (0.8 to 1.2 ml)	↓ 1.2 ml	NA
Shaking of blood tubes	↑ ↓	?
T-cell and APC count	?	?[d]
Transportation temp	?	↓
Incubation delay	↓ <30°C	?
Incubation time	No effect	?
Plasma separation delay	?[e]	NA
Plasma/PBMC storage	No effect	No effect
Analytical		
Within-run imprecision	↑ ↓	↑ ↓
Between-run imprecision	↑ ↓	↑ ↓
Between-operator imprecision	↑ ↓	↑ ↓
Between-laboratory imprecision	↑ ↓	↑ ↓
Automation	↓	↓
Immunological		
Boosting by TST	↑[f]	↑[f]
Modulation by PAMP	↑ ↓	?

[a] Republished from reference 1.
[b] APC, antigen-presenting cell, PAMP, pathogen-associated molecular pattern.
[c] An increase (↑) or decrease (↓) in the assay response or the response could go either direction (↑ ↓); ?, unknown effect; NA, not applicable.
[d] The mononuclear cell input is normalized.
[e] According to the manufacturer, a delay after plasma separation contributes to ELISA variability.
[f] Particularly in individuals infected with *M. tuberculosis*. Less proven in uninfected individuals.

reduction of the mitogen response below the assay threshold in the QFT-GIT assay (7–9). Jarvis et al. showed lower mitogen responses when QFT tubes are kept at room temperature (as suggested by the manufacturer) or lower temperatures, which may explain the higher indeterminate rate observed with incubation delay (10). The impact of processing delay (immediately after blood collection versus delayed) on the T-SPOT assay has not been studied in individuals with *M. tuberculosis* infection.

Beffa et al. showed a higher rate of T-SPOT indeterminate results during autumn and winter than in spring and summer, suggesting that transport of blood specimen at lower ambient temperatures may negatively impact the assay (11). The manufacturer of the T-SPOT assay offers the T-Cell Xtend reagent to extend processing time up to 32 h. The T-Cell Xtend reagent is an antibody complex added to the blood sample in the laboratory to deplete "inhibitory neutrophils." Although several groups have investigated the impact of the T-Cell Xtend reagent (12–14), none has compared immediate processing to delayed processing with and without the T-Cell Xtend reagent. Therefore, the potential utility of the T-Cell Xtend reagent remains to be demonstrated. This is crucial, because nearly all T-SPOT testing in the United States is currently outsourced (personal communication with laboratory directors), and thus, processing delay may lead to reproducibly false-negative results in a low-risk population (15).

The length of time T cells are stimulated with TB antigen in IGRAs can also vary between runs. According to package inserts, the length of incubation can range from 16 to 24 h for the QFT-GIT assay and 16 to 20 h for the T-SPOT assay. It is reasonable to expect longer incubations to give rise to higher concentrations of IFN-γ and larger numbers of SFC. However, Gaur et al. showed that incubation of QFT-GIT tubes for 16, 20, and 24 h does not increase TB response in infected individuals and does not cause false-positive results in uninfected individuals (5). Studies have also shown plasma storage at 4°C and −80°C for 4 weeks or PBMC storage at −80°C for 4 weeks does not have a major effect on QFT-GIT and ELISPOT results, respectively (6, 16).

Analytical sources of variability. Analytical sources of variability refer to unpredictable fluctuations in assay measurements due to uncontrolled factors in biological fluids (matrix effects); imprecision of pipetting; manipulation errors in centrifugation, decantation, and washing steps; and imprecision of measurement of final signal by the operator or the instrument (17). Much effort has been invested by the manufacturers to improve the analytical reproducibility of enzyme-linked immunoassays (17). Still, considerable analytical variability occurs, and clinical assays typically deal with this through adoption of an indeterminate range. To illustrate analytical variability with the QFT-GIT assay, we took a sample with a borderline-negative TB response (Fig. 2A) and another sample with a borderline-positive TB response (Fig. 2B) and measured TB response using an automated ELISA instrument over 20 consecutive ELISA runs. Not only did the TB response fluctuate quantitatively, giving a coefficient of variation (CV) of 14% and 11%, respectively, two (10%) and four (20%) times, respectively, they crossed the assay cutoff, giving rise to discrepant results.

Studies investigating between-run reproducibility of the QFT-GIT assay have shown that while there is a high degree of agreement between dichotomous results, there is considerable variability in quantitative results, which leads to discordant results when the TB response is bordering the assay cutoff (16, 18–20). Metcalfe et al. used a model to estimate between-run CV for 366 samples retested by two technicians after 2 or 3 days of storage at 4°C (18). They showed ±0.6 IU/ml (CV, 14%) between-run variability for all individuals and ±0.24 IU/ml (CV, 27%) variability for subjects with initial responses in the borderline range of 0.25 to 0.8 IU/ml. They reported a conversion and reversion rate of 9% and 7%, respectively; 86% (24/28) of discordant results occurred among samples with borderline TB response. Whitworth et al. reported between-laboratory standard deviation of 0.16 IU/ml when blood samples were tested in three laboratories using automated ELISA instruments (19). Discordant results were reported in 7.7%, 85.7% of which had responses within 0.25 IU/ml of the assay cutoff. Whitworth et al. also showed greater between-run variability when ELISA was performed manually versus automated (21). Tuuminen et al. measured within-run and between-run variability for the QFT-GIT and T-SPOT assays showing that between-run variability exceeded within-run variability for both assays (20).

Analytical error originating from ELISPOT and ELISA readers has been investigated for commercial IGRAs. Franken et al. assessed interreader variability for spot counting (22) with four dif-

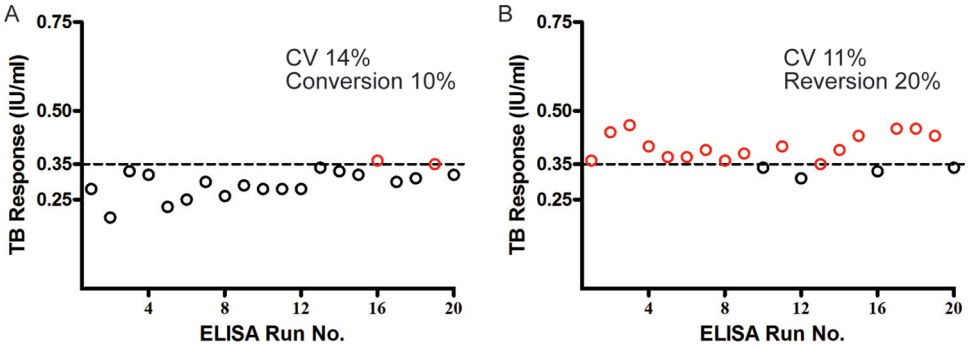

FIG 2 Between-run reproducibility of the QFT-GIT assay. (A and B) The TB response (IFN-γ concentration in antigen tube minus nil tube) was measured from a sample with a borderline negative result (A) and another sample with a borderline positive result (B) by an automated ELISA instrument over 20 consecutive runs. Samples were stored at 4°C between runs. Two to four ELISA runs were performed each day. The cutoff value for the standard QFT-GIT assay (dashed line) is shown for reference. Positive results are shown in red. Coefficient of variance (CV) and conversion and reversion rates are shown.

ferent observers and two automated readers. The proportion of positive results varied between readers by as much as 15%. Some individuals were found to systematically count more spots than others. In another study, van Zyl-Smit et al. reported no more than 1 spot variability at counts of 0 to 10 spots per well and no significant variability in optical density values for the ELISA plates reread over 2 h (23).

Postanalytical sources of variability. Clerical error during manual data entry can culminate in false results (19). Clerical error can be avoided by interfacing the ELISA system with the laboratory information system.

Manufacturing defects. Manufacturing defects can cause nonreproducible IGRA results. False-positive QFT-GIT results related to faulty antigen tubes have been documented. Slater et al. reported an incident of sudden increase in the daily QFT-GIT positivity rate from 10% to 31% (24). Paired testing of 463 subjects with the suspect antigen tube lot and a new lot confirmed a manufacturing defect causing false-positive results. Although the manufacturer could not identify the cause of the defect, in 2013, the manufacturer issued a market withdrawal for another faulty antigen tube lot causing false-positive results. Review of their manufacturing process identified a potential bacterial contamination. The manufacturer reported implementing corrective measures to prevent reoccurrence of this problem. Since then, there have been two other reports of faulty antigen tubes causing false-positive results (25, 26).

Indeterminate QFT-GIT results due to faulty mitogen tubes have also been reported. In 2013, the manufacturer of the QFT-GIT assay reported increased rates of indeterminate results with the introduction of a new lot of the mitogen tube. Interestingly, not all users were equally affected, which suggests that varied preanalytical practices, such as time to incubation, might have contributed to the problem.

Immunological sources of variability. To date, two immunological sources of IGRA variability have been reported: *in vivo* immune boosting by the TST and *ex vivo* immunomodulation by microbial products. van Zyl-Smit et al. and other investigators have reported a significant increase in TB response with the QFT-GIT and T-SPOT assays when these assays follow a TST by more than 3 days (27). This boosting effect can result in a conversion in a previously IGRA-negative individual. It is not clear how long the boosting persists and whether the purified protein derivative (PPD) formulation contributes to boosting. The mechanism of TST boosting is thought to be through an immune recall of memory T cells to RD1 antigens (and TB7.7 for QFT-GIT) that are present in the PPD extract. Although IGRA boosting is predominantly observed in *M. tuberculosis*-infected individuals (27), few studies have reported few conversions after TST testing in individuals with no risk factors for *M. tuberculosis* infection (28–31).

IGRAs are susceptible to modulation by endogenous and exogenous factors. Microbe-associated molecular patterns (MAMPs), such as peptidoglycan and lipopolysaccharide (LPS), are potent modulators of innate and adaptive immunity through activation of pathogen recognition receptors on immune cells (32). It is well-known that skin and environmental microorganisms can be introduced into the blood specimen during phlebotomy. It has also been shown that systemic circulation contains peptidoglycan originating from the gut microbiota (33). Thus, it is plausible that microbial contaminants and circulating MAMPs could modulate IGRAs to cause nonreproducible results. Gaur et al. showed that LPS added to QFT-GIT tubes at 10 ng/ml could nonspecifically increase the TB response and cause false-positive results (34). Gaur et al. also showed that as few as 10 CFU of *Staphylococcus aureus*, a skin colonizer, spiked into the antigen tube alone or into both the nil and antigen tubes is sufficient to cause false-positive results in 6.9% of 58 uninfected individuals tested (35). Although not shown in that study, it is possible that *S. aureus* could also cause false-negative results if added to the nil tube alone.

ELIMINATING SOURCES OF VARIABILITY

While variability caused by random sources is unavoidable and can be addressed only through adoption of an indeterminate range (variation attributed to random error), systematic sources of variability can be minimized through standardization of laboratory procedures and quality assurance of reagents. In the following sections, we recommend best practices for IGRAs based on lessons learned from reproducibility studies.

(i) Disinfection. IGRA package inserts currently lack specific instructions on the choice of disinfectant and the method of skin and tube septum disinfection to minimize introduction of microbial contaminants into the blood sample during phlebotomy. Standardize skin and tube septum disinfection, akin to that done for blood cultures.

(ii) Tube order. Standardize the order of the QFT-GIT tubes

during phlebotomy per the package insert (in the order purge tube, nil tube, antigen tube, and mitogen tube).

(iii) 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.

(iv) Tube shaking. Standardize gentle shaking of the QFT-GIT tubes per the package insert. Avoid separate shaking of the nil and antigen tubes, as differential shaking can result in a false-positive or false-negative result.

(v) 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 (8) or by using a portable incubator to transport the tubes from the field to the laboratory. Further studies are needed to determine whether the T-Cell Xtend reagent can prolong processing time for the T-SPOT assay.

(vi) Analytical error. Use automated ELISA and ELISPOT instruments to reduce analytical variability.

(vii) Manufacturing defect. Institute a quality assurance program to monitor positivity and indeterminate rates. When rates cross a preset threshold and persist (24), halt utilization of potentially faulty lots and alert the manufacturer.

(viii) 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 (27).

SUMMARY

IGRAs are functional assays that are susceptible to variability from various sources. Different sources of variability have different effects with the net sum of all sources resulting in either no change, increase, or decrease in assay response. The impact of variability sources becomes most noticeable when the TB response is bordering the assay cutoff. While systematic sources of variability can be minimized through assay standardization and quality assurance, random sources are unavoidable and can be addressed only through adoption of an indeterminate range, which is currently lacking for the QFT-GIT assay. Further studies are needed, particularly for the T-SPOT assay, to better define the extent of variability attributed to each source. With the launch of the newer QFT-Plus assay, there is a need for reproducibility data on the newer version, especially since additional tubes and mechanisms are involved.

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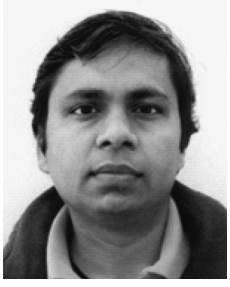

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