

Current Research in the Laboratory Diagnosis of Tuberculosis

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Published Date: May 20, 2016

INTRODUCTION

Tuberculosis (**TB**) is the main worldwide cause of mortality and morbidity due to bacterial infections and the second cause in terms of infectious agents, only preceded by the Human Immunodeficiency Virus (**HIV**). The etiologic agent of TB is the group of mycobacteria known as the Mycobacterium Tuberculosis Complex (**MTBC**). MTBC includes *M tuberculosis* (**MTB**), *M bovis*, *M africanum*, *M microti*, *M canetti*, and the *M bovis* vaccine strain also known as Bacillus Calmette-Guérin (**BCG**). Every member of the MTBC is pathogenic in humans while *M bovis* is usually pathogenic in the animal kingdom before invading humans. TB was declared a global health emergency by the World Health Organization (**WHO**) in 1993. Between 1990 and 2013, the tuberculosis mortality rate decreased by 45% due to worldwide efforts promoted by the WHO. According with reported statistics, TB leads to approximately 1.5 million deaths every year,

95% of which occur in low and middle-income countries, with an average number of 170 deaths per hour throughout the world. A third of the world's population is estimated to be infected with MTBC, with around 9 million cases developing annually.

Lung infection is the most frequent clinical form of TB and the most contagious, so it is a matter of primordial importance in terms of public health. The TB problem has worsened as a result of the appearance of MTB resistant to multiple drugs and due to the presence of HIV. In order to ensure an early diagnosis, one must be aware of individual and group TB risk factors and conduct rapid clinical evaluations and the appropriate diagnostic tests in symptomatic individuals with findings compatible with TB. Moreover, children are at greater risk of developing the disease if infected (i.e. within a few weeks to several months). The diagnosis of TB in children is a challenge in small children that are unable to expectorate and in whom conventional microbiological testing has low sensitivity.

MICROSCOPY

Although the current laboratory diagnostic method for TB in developing countries is based on microscopy (with a sensitivity of 20 to 80%) by identifying Acid-Fast Bacilli (**AFB**) with Ziehl Neelsen (**ZN**) staining, other more sensitive methods to identify MTB, particularly rapid molecular tests, are gaining acceptance due to their efficiency and applicability.

Fluorescence microscopy is more sensitive (10%) than ZN and fluorochrome-stained smears are quicker to process. However, its use is limited due to high costs resulting from the need for mercury vapor light sources, maintenance and the required dark room.

Light-Emitting Diodes

Light-Emitting Diodes (**LED**) provide the benefits of fluorescence microscopy without the associated costs. In 2009, the efficacy of LED microscopy was evaluated by the WHO. LED microscopy was developed to provide countries with limited resources the benefits of fluorescence microscopy. First, existing fluorescence microscopes have been converted into LED light sources. Compared with mercury vapor fluorescence microscopes, LED microscopes are less costly, require less energy and may also function with batteries; moreover, the lamps have a long half-life, there is no risk of releasing toxic products in case they break and they can be used in a lighted room. These qualities have made LED microscopy a viable alternative for use in settings with limited resources but providing their benefits (improving sensitivity and efficacy) were they are most needed.

SAMPLE DECONTAMINATION

Traditional Decontamination

Sodium hydroxide-based (NaOH) protocols including the Petroff method and NALC-NaOH, are broadly used in decontamination although NaOH may also compromise MTB viability; they can

kill up to 60% of bacilli in clinical samples. This may lead to false negative results, especially in paucibacillary infection cases such as those observed in HIV infected patients.

Chlorhexidine-based decontamination has been proven to be superior to the standard NALC-NaOH method.

Decontamination with Chlorhexidine

Mix equal volumes of 0.1% dithiotreitol (Sigma- Aldrich) and sputum sample for 10 min in a conical 50 mL tube. Then, add a volume of 0.7% chlorhexidine (Chlorhexidine digluconate, Sigma-Aldrich) to reach a final concentration of 100 mg / L. Vortex spin and incubate for 15 min at room temperature, shaking continuously. If inoculating in Columbia agar with 5% sheep blood (COS, bioMérieux), add 10 ml PBS, 0.6 g egg lecithin and 2 mL Tween 80 to the chlorhexidine decontaminated sample to inactivate the chlorhexidine, vortex and incubate for 5 min at room temperature. Add PBS to 40 mL, centrifuge at 1700 g for 15 min, discard the supernatant and resuspend the pellet in 0.5 mL sterile PBS, pH 6.8.

All patients, including children, suspected of harboring Pulmonary TB (**PTB**) and that are able to expectorate a sputum sample, should provide at least two sputum samples for bacilloscopy or one sample for Xpert® MTB / RIF [approved by the WHO] in a guaranteed quality laboratory. Patients at risk for drug resistance, HIV positive or severely ill, should undergo Xpert MTB / RIF as the initial diagnostic method. Serology testing in blood and Interferon Gamma Release Assays (**IGRA**) should not be used for the diagnosis of Active TB (**ATB**). **Table 1:**

Test	Site
Active tuberculosis diagnosis	
Sputum smear microscopy	Pulmonary TB
Nucleic acid Amplification tests (NAATs) [no-Xpert MTB/RIF]	Pulmonary TB and extra pulmonary TB
Xpert MTB/RIF	Pulmonary TB and extra pulmonary TB and RIF resistance
Automated liquid cultures and rapid species identification based on MPT64	Pulmonary TB and extra pulmonary TB; speciation

A combination of sputum microscopy and Xpert MTB / RIF may increase the diagnostic yield. Using Xpert MTB / RIF as a complement to a negative bacilloscopy has a sensitivity of 68% and a specificity of 99% compared to culture.

Immuno chromatography (MPT64) for MTBC identification in myco bacterial liquid culture has been added as a diagnostic method for the rapid diagnosis of TB.

Fluorescence Microscopy (**FM**), in which auramine-based staining makes AFB fluoresce against a dark background, is also widely used in several parts of the world. Compared to ZN and considering culture as the gold standard, FM is the most sensitive method.

CULTURE MEDIA

Lowenstein-Jensen

In spite of important advances in the molecular diagnosis of TB in the last two decades, culture remains the universal gold standard for laboratory diagnosis, since it allows post-culture antimicrobial sensitivity testing and genotypification. It generally takes 3 to 8 weeks, with a sensitivity of 100 colony-forming units per milliliter (CFU / mL). Traditional culture methods use solid media such as Lowenstein-Jensen (**LJ**) and Ogawa and can be performed locally.

Isolate identification in culture media and TB sensitivity tests add 2 to 3 weeks more to the time period required to establish a definitive TB diagnosis. Liquid culture is more sensitive and rapid than solid.

Liquid Media

MTB are fastidious organisms that are routinely detected only after 7-12 days using commercially available advanced automated systems. These robots use different Middlebrook liquid culture media formulations and decrease diagnostic delays in MTB detection compared to inoculation in standard solid media such as LJ.

MTB can be cultured from the respiratory tract for the diagnosis of Pulmonary Tuberculosis (**PTB**). In these samples, the excessive growth of contaminants including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* make difficult MTB isolation and culture. Various protocols have been recommended to limit the excessive growth of contaminants, including the addition of antibiotics and antifungal agents to the culture medium. Several decontamination methods have been developed for various MTB culture media. The Myco Bacterial Growth Indicator Tube (**MGIT**) has a rapid and sensitive culture medium and low contamination rates. However, its high cost is prohibitive in settings with limited resources. The BACTEC 960 liquid culture system uses PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) to curb contamination. However, these protocols have been shown to inhibit MTB viability to a certain extent.

New Media

In ordinary blood agar medium, Mycobacterium species can grow, except for Mycobacterium ulcerans, in a cost-effective manner and contamination is not an issue if decontamination procedures are strictly followed. The sample is inoculated in 5% sheep blood agar in inclined tubes and incubated at 37°C. Blood agar should be considered the first-line medium for Mycobacterium species culture. It saves time; it is cost-effective and as sensitive and fast as automated methods. This is of particular relevance in countries with limited resources in which TB Prevalence Is High.

The culture medium can be optimized, beginning with the blood media. Egg lecithin (MOD4 medium), an antidote to the chlorhexidine used to decontaminate samples, has growth promoting properties. It improves growth in solid media, in a microaerophilic atmosphere compared to normal oxygen tension.

There is a new solid culture medium formulation named MOD9. It was developed in order to further improve culture sensitivity and preclude the need for blood in this type of culture medium. MOD9 was derived from MOD4 in which 5% sheep's blood is eliminated as well as heat-inactivated 15% bovine serum but 15% heat-inactivated lamb's serum is added (Life Technologies, Saint Aubin, France). Also, 100 mg / L ascorbic acid are added to mimic a microaerophilic environment. A mixture of Azorubine and Ponceau 4R, normally used as food coloring (Sainte-Lucie, Gouvieux, France) to stain the medium red and increase the contrast of white-yellowish mycobacteria, was also added. The culture medium is poured in 55-mm sterile Petri dishes (Gosselin, Borre, France). Culture media can be observed at plain sight every 24 h in search of colonies, for four weeks; time to detection is 10 days in MOD9 versus 17 days in LJ and both MTB and Non-Tuberculosis Mycobacteria (**NTM**) can be grown with lower contamination rates. The combination of chlorhexidine decontamination in MOD9 and the standard NALC-NaOH / BACTEC MGIT routinely used protocol have also been compared. The chlorhexidine/MOD9 protocol has been proposed for the diagnosis of MTB and NTB as an alternative or complement to the MGIT protocol. This is still undergoing further evaluation.

Intrinsic fluorescence (auto fluorescence) is a characteristic of some microorganisms that permits their detection without the need for fluorescence staining. Mycobacteria, including MTB, emit fluorescence in the cyano range of the visible spectrum.

Micro colonies are detected by observing MTB auto fluorescence at 12.5 X power and between 450 nm and 550 nm, in a fluorescence microscope and a camera with a GFP filter. Readings begin 24 h after inoculation and then every 12 h until day 20 after inoculation. Auto fluorescence is detected by microscopy, in plain sight.

With the combination of an appropriate medium, optimizing incubation conditions and using powerful detection tools, primo-isolation can be obtained with sputum microculture techniques (MODS) in 4.7 days compared to 12.5 days when using BACTEC MGIT. For further simplification, ascorbic acid is added as an antioxidant, thus permitting growth in normal oxygen tension conditions. The primary culture of clinical samples grew in 4.2 days in a microaerophilic atmosphere, and in 4.3 days in solid medium complemented with 100 mg / L ascorbic acid and a normal atmosphere.

Therefore, MTB culture could progress rapidly between clinical sample isolation until antibiotic testing by modifying current methods. As a matter of fact, the isolation of MTB XDR from a sputum sample in 72 hours and the determination of its resistance to rifampicin could be determined within another 36 h. Moreover, testing antibiotic sensitivity in parallel to the primary inoculation may decrease the time to diagnosis to 72 h in ZN positive patients. Any antibiotic could be tested with this assay in order to discover new resistances. We must point out that this culture strategy can be adapted in countries with limited resources. This MTB culture optimization is a reflection of the renaissance of microbial culture and the need to continue investing in this field.

Conversion of the sputum culture in two months on solid media is the best established predictor of therapeutic response.

DNA AMPLIFICATION

A microbiological diagnosis can only be confirmed by MTBC culture or the identification of specific nucleic acid sequences in a sample obtained from any diseased site.

Methods based on DNA amplification can overcome the delays caused by the need to culture sufficient biomass. The polymerase Chain Reaction (**PCR**) has been shown to play an important role as an alternative diagnostic tool in developed countries and has yielded variable results, with sensitivities ranging between 42% and 100% and specificities between 85% and 100%; various targets have been used in PCR such as 6110, 65 kDa, TRC4, GCRS, 16S, to mention a few. Direct molecular MTBC detection is recommended because it is a sensitive and specific method, and PCR should be used as an adjunct to other laboratory TB diagnostic methods.

The cost-effectiveness of direct microscopic examination in the rapid diagnosis of PTB has yet to be proven. Real-time PCR is more costly than direct microscopic examination (0.57 \$ for microscopy after Ziehl-Neelsen staining vs. 19.56 \$ for PCR), but it is more cost-effective if treatment costs are included (412 dolls? for microscopy after Ziehl-Neelsen staining vs. 382 for PCR).

PCR allows for the rapid and exact identification of MTB in PTB directly in positive liquid culture (MB/BacT) and it correlates with FAB cord formation, an important technological advance in clinical mycobacteriology.

PCR is also useful in the fast and reliable diagnosis and is an important tool against TB when used in conjunction with available clinical data. It has adequate efficiency in the rapid molecular diagnosis of Extra Pulmonary TB (**ETB**), but it improves when performed in urine when compared to CSF. The PCR result has an acceptable impact on the management of these patients.

PCR-based sequencing is considered the gold standard methodology for mycobacterium identification. PCR is performed and followed by amplicon sequencing in an automatic sequencer. Laboratory TB diagnostic tests have rapidly evolved and now provide a sensitive and specific diagnosis of MTB and its drug sensitivity. Some of the molecular tests have been incorporated to routine laboratory testing allowing physicians to promptly begin the required drug regimens.

Xpert MTB/RIF

This technique has an excellent performance in the detection of MTB and rifampicin resistance. Sensitivity is estimated at 98% in AFB positive samples and 68% in negative bacilloscopies.

Based on evidence, the WHO recommends that Xpert MTB / RIF:

- Should be used in lieu of microscopy, culture and drug sensitivity testing as an initial diagnostic test in individuals suspected of harboring multi-drug resistance (MDR) or HIV-associated TB;

- It can be used as a microscopy follow-up test in adults in whom TB and HIV are less concerning, especially in subsequent testing of bacilloscopically negative samples;
- It can be used in lieu of conventional microscopy and culture as an initial diagnostic test in adults suspected of harboring TB;
- It must be used instead of microscopy, culture and drug sensitivity testing as the initial diagnostic test in children suspected of harboring MDR or HIV-associated TB;
- It may be used in lieu of conventional microscopy and culture as an initial diagnostic test in children suspected of harboring TB.

The use of Xpert MTB / RIF does not preclude the need for conventional microscopy, culture or drug sensitivity testing required to control therapy and for the detection of resistance to drugs other than rifampicin.

In patients suspected of harboring PTB and a negative FAB, the Xpert MTB / RIF and / or sputum culture must be performed. In patients with negative FAB and Xpert MTB / RIF, but with solid clinical evidence of TB, anti TB treatment should only be initiated after obtaining samples for culture.

A positive Xpert MTB / RIF decreases the time to diagnosis and to the initiation of appropriate treatment, with a possible economic savings.

The diagnosis of TB in children is based on an exhaustive evaluation of all the evidence obtained after a careful exposure history, physical examination and other pertinent facts. Although most children with TB have pulmonary involvement, they also commonly present paucibacillary disease with no evident pulmonary cavitations but that frequently spreads to intrathoracic lymph nodes.

In all children suspected of harboring intrathoracic TB (i.e., pulmonary, pleural, mediastinal or in hilar lymph nodes), bacteriological confirmation should be obtained by examining respiratory secretions (expectorated sputum, induced sputum, gastric lavage) by bacilloscopy, Xpert MTB / RIF, and / or culture. Table 2.

Table 2: Diagnostic guidelines for TB in children.

1. Careful history (including prior contacts and symptoms consistent with TB)
2. Tuberculin test
3. Chest X-ray, if available
4. Bacteriological confirmation, if possible

In children, the risk of TB increases when an active case (infectious, TB with positive bacilloscopy) is present in the same household or when the child is malnourished, is infected by HIV or has recently had measles. The WHO's Integrated Management of Childhood Illness (**IMCI**) that is widely used in primary care in low-income countries has established that TB should be considered in any child with:

- Unexplained weight loss or failure to thrive;
- Unexplained fever, particularly if it lasts over 2 weeks;
- Chronic cough;
- Exposure to an adult with probable or definitive infectious pulmonary TB.

INTERFERON GAMMA RELEASE ASSAY

In most individuals, the initial infection by MTB is eliminated or contained by the host's immunity and the infection remains latent. The identification and treatment of LTBI may decrease the risk of disease and are strategies for TB control, particularly in low incidence settings. Diagnostic tests for TB infection are used worldwide to detect individuals infected with latent TB (**LTBI**) and prevent dissemination; however, there is no diagnostic gold standard. The purpose of testing in LTBI is to identify individuals at risk of developing ATB; these individuals benefit most from treatment (also known as prophylaxis). Therefore, only these should be tested and treated if the test is positive.

There are currently two types of tests to screen individuals with LTBI. These are the Tuberculin Skin Test (**TST**) and two different assays that measure Interferon Gamma Release (**IGRA**) in patient lymphocytes after exposure to specific TB antigens.

The TST was first developed by Charles Mantoux in 1907 and has been an important factor in the decrease of TB in the greater part of the western world. It has existed in different forms but currently, Mantoux's test is used as the intradermal injection of 5 TU derived from the Purified Protein (**PPD**) or 2 U of PPD-RT23.

The diagnosis of LTBI is complex and the test used for many years is the TST that reflects a state of hypersensitivity after previous contact with MTB, by producing local skin induration surrounded by erythema; TST is obtained from the filtration of Koch's bacillus cultures, which is subsequently sterilized and concentrated. The test is limited by the fact that it is also constituted by other mycobacteria such as *M bovis*, or *M avium*, which can lead to false positive results; this may also occur following vaccination with the Calmette-Guérin bacillus (**BCG**). It is easy to infer that the result of the TST reflects a state of hypersensitivity in immune suppressed individuals such as those infected by viruses, undergoing immunosuppressive treatment, vaccinated with live viruses and those in the extremes of life, in whom the tests may yield false positive results; this may also be due to inadequate storage, inadequate inoculation or the erroneous interpretation of induration in the test site. However, this test should be a priority in individuals in whom the result would modify treatment or therapeutic interventions as it is undoubtedly an excellent ancillary method in the diagnosis of LTBI.

More distressing is the fact that false negative results may occur in patients with ATB, HIV, immune suppression, malnutrition or under treatment with immune suppressors. Unfortunately, those individuals in whom TST has limited sensitivity are those at greatest risk of progression to

active disease if they become infected. Since there is no gold standard for LTBI diagnosis, sensitivity and specificity are estimated using substitute reference standards. Sensitivity is estimated among cases of TB confirmed by culture, and specificity is deduced among individuals at low-risk and with no known previous exposure to TB, in low incidence settings. The determination of IGRA sensitivity and specificity in children is more difficult. Most children with clinical TB have no microbiological confirmation, so they lack absolute proof of infection which is necessary to evaluate the assay's sensitivity; instead, less reliable clinical diagnostic methods have been used.

The immune response is pivotal for active infection implantation and is also very important in LTBI since activation of the immune cytokine cascade is crucial to the development of tuberculosis. One of the key molecules for infection control is interferon- γ (INF- γ), produced when *Mycobacterium tuberculosis* is transmitted by air via Pflügge droplets; the infected individual develops an inflammatory response in which macrophages phagocytose bacilli and release cytokines that attract neutrophils, macrophages and T lymphocytes that in turn, secrete tumor necrosis factor alpha (TNF- α) and INF- γ . In synthesis, this is what constitutes a tuberculosis infection. Briefly, INF- γ is produced by CD4+, CD8+ T lymphocytes and natural killer (NK) cells; INF- γ activates the infected macrophages that produce interleukin-1 (IL-1) and TNF that are released to limit mycobacterial growth and multiplication. Although INF- γ production is insufficient to control tuberculosis, its effect is essential to the protective immune response when faced by this microorganism Figure 1.

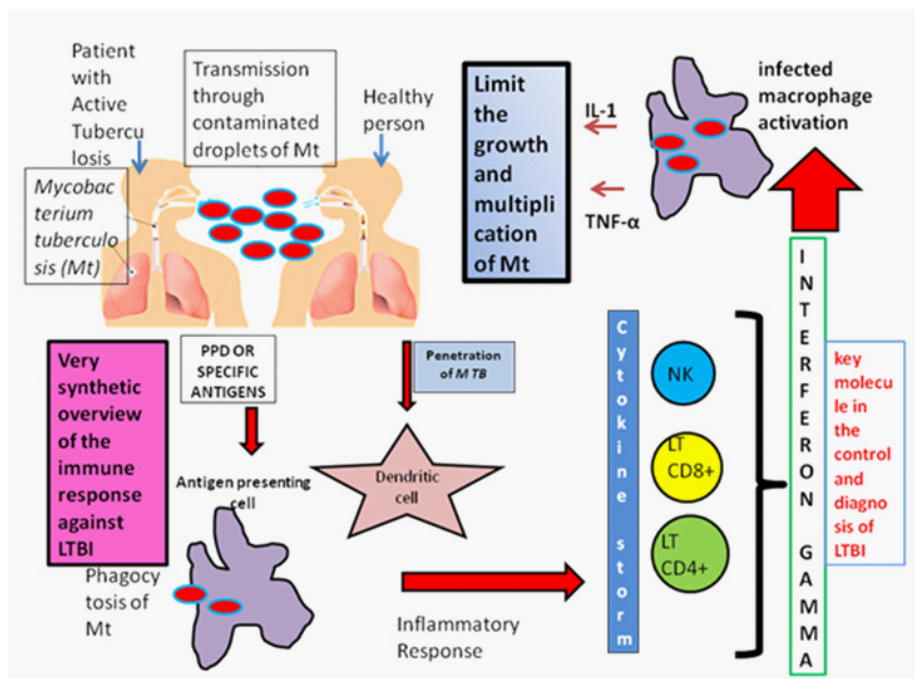


Figure1: The figure shows very schematically the immune response against TB infection, highlighting the role of interferon gamma in control and diagnosis of LTBI.

Recently, two new assays have been introduced after Food and Drug Administration (FDA) approval. They detect a specific immune response *in vitro* against MTB. These tests are QuantiFERON-TB Gold In-Tube (Cellestis / Qiagen, Carnegie, Australia) and the T-SPOT. TB analysis (Oxford Immunotec, Abingdon, U.K). The first available assay to detect MTB infection (QuantiFERON-TB) was approved by the FDA in 2001. Subsequently in 2005, the new QFT Gold In-Tube version was introduced, simplifying the addition of TB specific antigens.

As in this first assay, this new version detects specific MTB immune responses in blood. Three specific MTB antigens are used: the early secretory antigenic target 6 (ESAT-6), culture filtered protein 10 (CFP10) and protein TB7.7.

A positive result for MTB infection will yield an IFN γ response against TB antigens above the cutoff value (background IFN γ response in negative control). These assays are designed to detect a specific cellular immune response against MTB antigens.

The positive predictive value of both TST and TB IGRA are related to the prevalence of TB in the test population. Therefore, there may be false positive results with both tests. Both TST and IGRA are acceptable but imperfect tests for LTBI, with inherent advantages and disadvantages. Table 3.

Table 3: Comparison of the Tuberculin skin test and Interferon Gamma Release Assay.

Characteristic	TST	IGRA
Advantages	Simple Requires no equipment Can be performed by trained HCW at remote sites	24-48 h No cross-reaction with BCG Less cross-reaction than TST with NTM
Risks	False negatives False positives 48-72 h	Blood drawn Exposure to blood pathogens Decreased adverse events
Resources	Less expensive than IGRA Requires no laboratory Adequate storage	More expensive than TST Equipped laboratory Qualified personnel

HCW: Health Care Worker; NTM: Nontuberculous Mycobacteria

CONCLUSION

ATB and LTBI are found throughout the world, affecting approximately a third of the human population. TB is transmitted from person to person when speaking, coughing, sneezing or after direct contact with other individuals. Although infected with TB, individuals may be asymptomatic. Many TB patients do not seek medical care and will unfortunately transmit the infection to others during this infection period.

Mycobacteria are environmental organisms that can act as opportunistic pathogens and with few exceptions, will lead to severe infections including TB (**MTBC**), leprosy (*M leprae*), and Buruli ulcer (*M ulcerans*). Mycobacterial cultures were always considered to require specialized laboratories.

As is usual in medical microbiology, MTB culture is the aim of microbiologists for the diagnosis of PTB, in the hope of detecting viable vs. dead microorganisms, describe the complexity of mixed infections and allow advanced “omic” scale studies. Current techniques can now provide a culture-based diagnosis in less than 48 h under optimal conditions.

Screening tests for patients that may harbor LTBI have recently changed. Mantoux’s tuberculin skin test was introduced in the decade of the 1890’s as a skin test to determine whether an individual was infected with TB. Both TST and TB IGRA are valuable methods for LTBI detection. An advantage of TB IGRA is that the patient need not return for test interpretation, unlike TST.

Both TST and IGRA are acceptable but imperfect tests for LTBI diagnosis, with advantages and disadvantages (Table 3). IGRA offers some advantages over TST. The primary objective of IGRA is the identification of those individuals that will benefit from LTBI therapy. Unfortunately, IGRA (and TST) are limited by their inability to distinguish reactivation from reinfection, their precision is decreased in immunocompromised patients, and they are unable to discriminate different stages in the LTBI spectrum.

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