Let's Talk TB

A Series on Tuberculosis, A Disease That Affects Over 2 Million Indians Every Year

Diagnosis of Tuberculosis: Importance of Appropriate Specimen Collection

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Abstract

A good diagnostic approach for TB requires collection of the right clinical specimen(s) of adequate quality and quantity. For pulmonary TB, sputum is the most important sample for laboratory testing. Although blood is a popular sample in the Indian private sector, there is no accurate blood test for active TB. For extra-pulmonary TB, it is critical to obtain specimens from the site of disease, and this usually includes collection of tissue (biopsy) and/or body cavity fluids from the suspected disease site. For childhood TB diagnosis, sputum can be collected from older children. In young children, fasting gastric aspirates are the routinely collected samples. For latent TB infection diagnosis, there are two main options – interferon-gamma release assays which require venous blood samples, or the tuberculin skin test (Mantoux), which is an intra-dermal skin test. In all the above situations, clear instructions on specimen collection should be provided to patients as well as to laboratories and clinics. Quality of specimens can often have a big impact on test results, and every effort should be made to ensure quality in specimen collection, transport and processing.

Key words: tuberculosis; diagnosis; sputum specimens; sample collection methods

INTRODUCTION

In a previous article in this series, the best practices for pulmonary tuberculosis (TB) diagnosis were described.[1] This article provides additional information on specimen collection for laboratory diagnosis. A good diagnostic approach for TB requires collection of the right clinical specimen and use of the appropriate laboratory test. All clinicians, therefore, should have basic knowledge about the types of specimens that must be collected, and should be able to provide clear instructions to their patients on how to provide such specimens at the laboratory, or in the clinic, if samples are collected and then transported to laboratories.

The specimen type is decided by the site of disease, purpose of testing or the patient population. The following four distinct sites/purposes should guide specimen collection (**Table 1**):

- Active, pulmonary tuberculosis
- Active, extra-pulmonary tuberculosis (multiple sites are possible)
- Childhood tuberculosis
- Latent tuberculosis infection

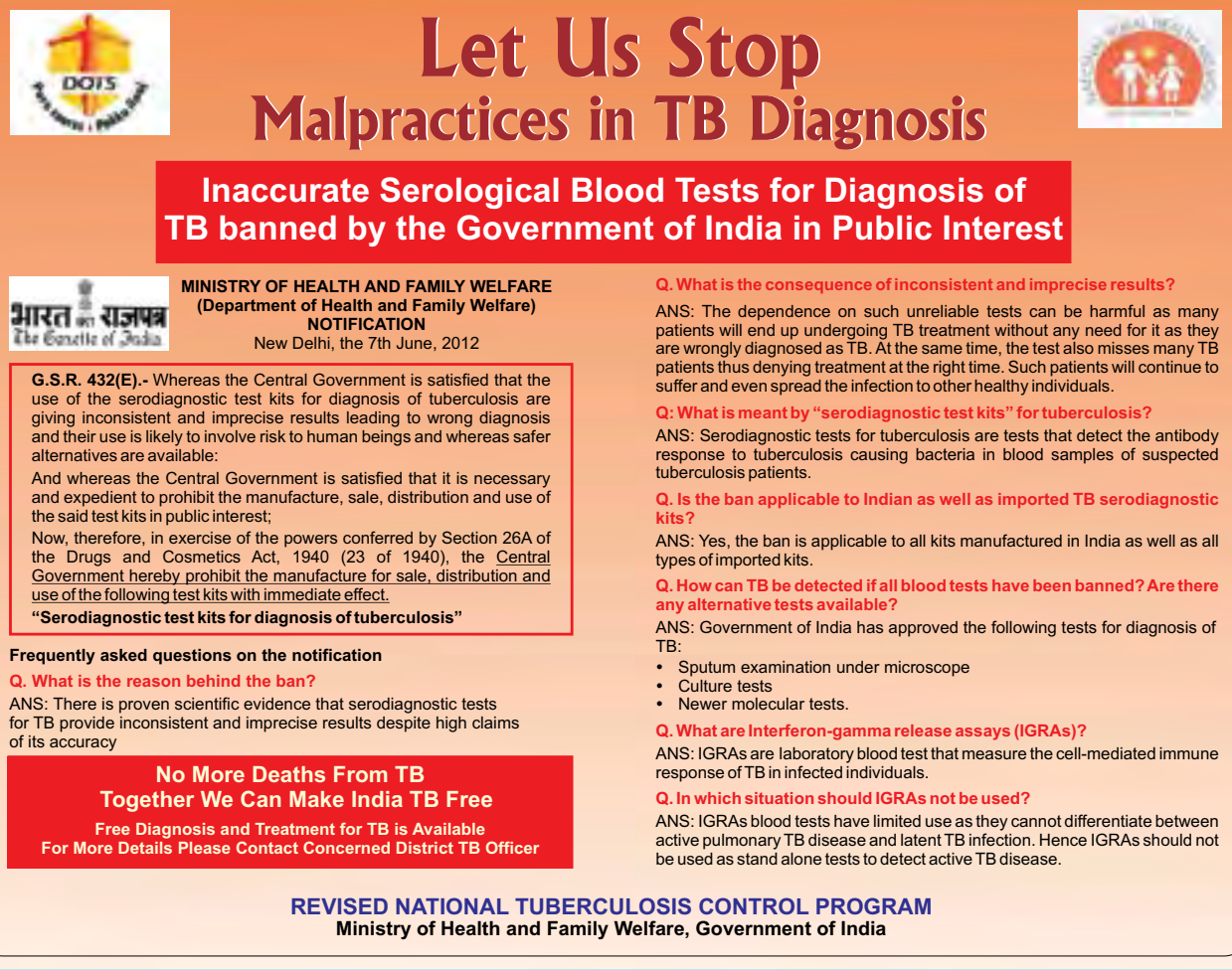
SPECIMENS FOR ACTIVE, PULMONARY TB

For pulmonary TB, sputum is the single most important sample for laboratory testing. For several reasons, blood is a popular sample in the Indian private sector.[2] Unfortunately, there is no accepted, valid blood test for pulmonary TB. Blood is not the ideal sample for any of the accepted TB testing technologies, namely, smear microscopy for acid-fast bacilli (AFB), culture, and molecular tests (e.g., polymerase chain reaction [PCR]). In fact, blood-based, serological, antibody-detection tests for TB (e.g., IgM/IgG anti-

Table 1 – The specimen type is decided by the site of disease or purpose of testing or patient population

Site, purpose, or patient population	Specimen of choice	Comments
Active, pulmonary TB	Sputum (spontaneous or induced)	- Sputum must be produced deep from within the lungs - Saliva is not acceptable - At least two sputum samples must be collected - Blood is not acceptable as a sample for active, pulmonary TB - Rarely, bronchoalveolar lavage (BAL) is used to collect lung secretions – this requires expertise and hospital care
Active, extrapulmonary TB		
TB lymphadenitis	Lymph node aspirate or biopsy	- Requires needle aspiration and/or excision biopsy - Samples are then sent for smears for AFB, liquid culture, molecular (PCR) tests, and histopathological examination - Histopathology and liquid culture are the most important tests; PCR may help, if positive
Pleural effusion (TB pleuritis)	Pleural fluid and pleural biopsy	- Requires pleural tap and/or biopsy - Samples are then sent for pleural fluid analysis, smears for AFB, liquid culture, molecular (PCR) tests, and histopathological examination; pleural fluid adenosine deaminase (ADA) or interferon-gamma is often helpful - Histopathology and liquid culture are the most important tests; PCR may help, if positive
Ascites (abdominal TB)	Ascitic fluid and peritoneal biopsy	- Requires ascitic tap and/or biopsy - Samples are then sent for smears for AFB, ascitic fluid analysis, liquid culture, molecular (PCR) tests, and histopathological examination; ascitic fluid ADA or interferon-gamma is often helpful - Histopathology and liquid culture are the most important tests; PCR may help, if positive
TB meningitis	Cerebrospinal fluid (CSF)	- Requires spinal tap for CSF collection - Samples are then sent for smears for AFB, CSF analysis, liquid culture, molecular (PCR) tests - Liquid culture of CSF along with CSF analysis is most important; PCR may help, if positive
Bone and joint TB	Bone/synovial tissue via biopsy	- Histopathology and liquid culture are the most important tests; PCR may help, if positive
Urinary tract and kidneys TB	Urine and tissue via biopsy	- Histopathology and liquid culture are the most important tests; PCR may help, if positive
Genito-urinary tract TB	Tissue via biopsy (e.g., endometrial tissue in women)	- Menstrual blood is not ideal; it is important to collect endometrial tissue - Histopathology and liquid culture are the most important tests; PCR may help, if positive
Childhood TB	Sputum in older children; in younger children, gastric aspirates	See Table 2 for additional options and comments
Latent TB infection	Whole blood for interferon-gamma release assays (IGRAs); or Mantoux intra-dermal skin test	- IGRAs are only meant for latent TB infection – they cannot separate latent infection from active disease - Mantoux skin test must be correctly performed and read

Box 1. Frequently asked questions about the Indian governmental ban on TB serological tests, published in several Indian newspapers in December 2012 in India



Let Us Stop
Malpractices in TB Diagnosis

Inaccurate Serological Blood Tests for Diagnosis of TB banned by the Government of India in Public Interest

भारत का राजपत्र
The Gazette of India

MINISTRY OF HEALTH AND FAMILY WELFARE
(Department of Health and Family Welfare)
NOTIFICATION
New Delhi, the 7th June, 2012

G.S.R. 432(E).- Whereas the Central Government is satisfied that the use of the serodiagnostic test kits for diagnosis of tuberculosis are giving inconsistent and imprecise results leading to wrong diagnosis and their use is likely to involve risk to human beings and whereas safer alternatives are available:

And whereas the Central Government is satisfied that it is necessary and expedient to prohibit the manufacture, sale, distribution and use of the said test kits in public interest;

Now, therefore, in exercise of the powers conferred by Section 26A of the Drugs and Cosmetics Act, 1940 (23 of 1940), the Central Government hereby prohibit the manufacture for sale, distribution and use of the following test kits with immediate effect.

"Serodiagnostic test kits for diagnosis of tuberculosis"

Frequently asked questions on the notification

Q. What is the reason behind the ban?

ANS: There is proven scientific evidence that serodiagnostic tests for TB provide inconsistent and imprecise results despite high claims of its accuracy

No More Deaths From TB
Together We Can Make India TB Free
Free Diagnosis and Treatment for TB is Available
For More Details Please Contact Concerned District TB Officer

Q. What is the consequence of inconsistent and imprecise results?

ANS: The dependence on such unreliable tests can be harmful as many patients will end up undergoing TB treatment without any need for it as they are wrongly diagnosed as TB. At the same time, the test also misses many TB patients thus denying treatment at the right time. Such patients will continue to suffer and even spread the infection to other healthy individuals.

Q: What is meant by "serodiagnostic test kits" for tuberculosis?

ANS: Serodiagnostic tests for tuberculosis are tests that detect the antibody response to tuberculosis causing bacteria in blood samples of suspected tuberculosis patients.

Q. Is the ban applicable to Indian as well as imported TB serodiagnostic kits?

ANS: Yes, the ban is applicable to all kits manufactured in India as well as all types of imported kits.

Q. How can TB be detected if all blood tests have been banned? Are there any alternative tests available?

ANS: Government of India has approved the following tests for diagnosis of TB:

- Sputum examination under microscope
- Culture tests
- Newer molecular tests.

Q. What are Interferon-gamma release assays (IGRAs)?

ANS: IGRAs are laboratory blood test that measure the cell-mediated immune response of TB in infected individuals.

Q. In which situation should IGRAs not be used?

ANS: IGRAs blood tests have limited use as they cannot differentiate between active pulmonary TB disease and latent TB infection. Hence IGRAs should not be used as stand alone tests to detect active TB disease.

REVISED NATIONAL TUBERCULOSIS CONTROL PROGRAM
Ministry of Health and Family Welfare, Government of India

dawp 17137/13/0001/1213

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bodies using ELISA and rapid tests) have been strongly discouraged by the World Health Organization (WHO), and banned in 2012 by the Government of India.[3]

Although there are blood-based tests for latent TB infection (e.g., interferon-gamma release assays such as QuantiFERON TB-Gold – marketed in India as "TB Gold" by Qiagen), these tests have no value for active pulmonary TB diagnosis and should be avoided.[4,5] Recently, the Revised National TB Control Programme (RNTCP) in India published frequently asked questions (FAQs) on the ban on serology in leading Indian newspapers (**Box 1**).

All patients (adults, adolescents, and older children who are capable of producing sputum) suspected of having pulmonary TB should have at least two sputum specimens submitted for microscopic examination and/or a World Health Organization (WHO) approved molecular test (e.g., Xpert MTB/RIF by Cepheid Inc.) or culture (e.g., MGIT by BD Diagnostics).[6] To reduce drop-outs, where feasible, 2 sputum specimens can be collected on the same day, a minimum of one hour apart.[7] Earlier recommendations required the collection of 3 sputum samples, but the current policy requires 2 specimens for microscopy, provided microscopy is done with quality assurance.

All specimens must be collected in sterile, leak-proof, laboratory-approved containers, labeled on the side with the patient's name and the date of collection

Figure 1 – Sputum samples are usually thick and mucoid with varying color

and accompanied by a carefully completed requisition form providing the patient's name and age, the physician's name and address, the date and time of collection, whether the specimen is diagnostic or follow-up and the specimen type and site. As much as possible, specimens collected for initial diagnosis should be obtained before the initiation of anti-TB therapy.

Sputum can be collected in one of two ways: spontaneous expectoration, and sputum induction. Spontaneous expectoration requires the patient to cough up a sputum sample from the lung without any assistance. If the patient is unable to produce a sample, then sputum induction can be done to stimulate a deep cough. Sputum induction is usually done by asking the patient to inhale nebulised hypertonic saline in a sputum induction chamber that ensures biosafety. Sometimes, bronchoalveolar lavage (BAL) may be necessary to collect samples from the lung. BAL is performed in a hospital with the patient sedated. A 5 ml portion of the lavage fluid may be submitted for TB testing and a post-bronchoscopy sputum specimen should also be collected and submitted.

Sputum is a respiratory secretion originating from deep within the lungs. Unless properly instructed, patients may provide saliva samples instead of sputum. Thus, all patients should be instructed on the difference between sputum and saliva or nasopharyngeal secretions and the necessity for a deep, productive cough. The following instructions should be provided to patients:[8]

- The importance of sputum examination for diagnosis or follow-up of pulmonary TB;
- The fact that TB is usually a curable disease and that if their test is positive they will receive treatment (available free anywhere in India via the RNTCP);
- How to open and completely close the screw-capped containers without touching the inside;
- The need for collecting 1 to 2 teaspoons of real sputum, not saliva;
- To first wash their mouth out with cooled, boiled water to avoid food particles in the specimen;
- How to produce good sputum (i.e., the patient is relaxed and seated. They cover their mouth with a tissue and after several repeated deep inhalations and exhalations of breath they cough the sputum up into their mouth from as deep inside the chest as possible. They then carefully spit the sputum into the opened container and close it);
- If possible, to collect the first specimen in the early morning since sputum and bacilli accumulate in the lungs overnight;
- To expectorate sputum specimens in the open air or in a well ventilated area;
- How to avoid contamination of the exterior of the container (i.e., by carefully spitting and closing the container);
- To wash their hands with soap and water after specimen collection;
- How to safely deliver the morning sputum to the laboratory as soon as possible after it is produced; and
- The need for at least two sputum specimens to facilitate diagnosis.

A good specimen should be approximately 5 ml. All samples should be inspected by clinic staff before sending to the laboratory. Sputum is usually thick and mucoid and color may vary (white, green, or bloody) (**Figure 1**). It may be fluid and contain pieces of purulent material. Clear saliva or nasal discharge is not a suitable specimen (**Figure 2**). Induced sputum samples are usually watery, but are acceptable since they come from the lungs. The accompanying form should state 'induced sputum'. Specimens should be equal in volume to about two teaspoons of material. If the specimen is inadequate the patient must be asked to repeat the procedure until an adequate quantity and quality of specimen is obtained.

Suitable sputum containers should be wide-mouthed, sterile, disposable, translucent and leak-proof with a screw cap and a space for labeling on the side. Alternatively, if the specimen is for smear and culture, it can be expectorated directly into a sterile 50 ml conical, screw-capped laboratory tube. Re-usable glass, screw-capped universal containers may be used if the laboratory has a facility for sterilising and cleaning the vials for re-use. All containers must be labeled with the patient's name and the date of specimen collection in indelible ink on the side of the container, not on the cap.

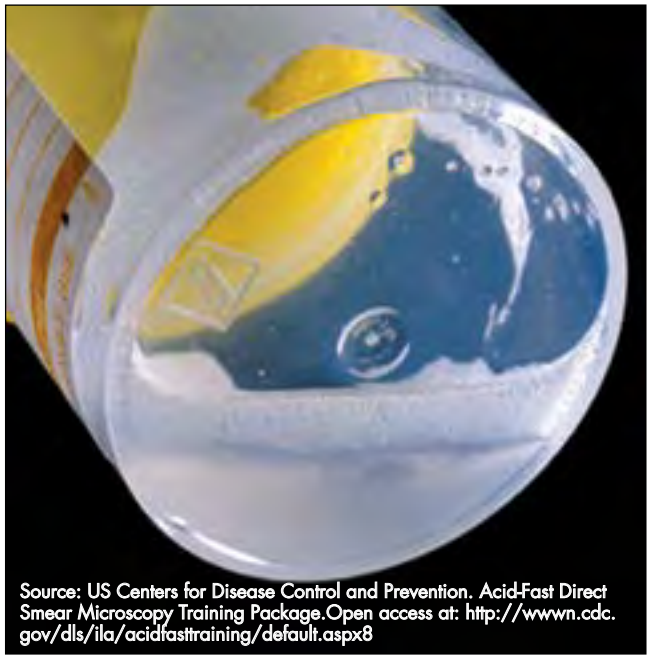
Source: US Centers for Disease Control and Prevention. Acid-Fast Direct Smear Microscopy Training Package.Open access at: http://wwwn.cdc.gov/dls/ila/acidfasttraining/default.aspx8

Figure 2 – Saliva is not an acceptable specimen for TB diagnosis

Sputum samples must be collected and transported safely so as to avoid the risk of infection to clinic and lab staff or other handlers. Containers must be capped firmly and any sputum noticed on the outside of the container must be wiped clean with bleach. Specimens should be packed upright in accordance with national requirements for transportation. Forms must be kept separate from the specimens to avoid contamination. Specimens should be transported to the laboratory as soon as possible and if there is a delay of > 2hours, they should be refrigerated.

The diagnosis of multi-drug resistant TB (MDR-TB) is usually based on molecular tests (e.g., Genotype MTBDRplus by Hain LifeScience; or Xpert MTB/RIF by Cepheid) or liquid cultures (e.g., MGIT by BD or BacT Alert by bioMerieux) done on sputum samples.

SPECIMENS FOR ACTIVE, EXTRA-PULMONARY TB (EPTB) DIAGNOSIS

EPTB can occur in many sites, the most common sites being lymph nodes, pleural, abdominal and meningeal sites. Other sites can include bone and joints, kidneys, genitourinary tract, and pericardial. EPTB cannot be diagnosed with sputum or blood specimens. It is critical to make an effort to collect tissue and fluids from the site of the disease. This may require surgical expertise and referral to a center where biopsies can be done safely. **Table 1** shows the various types of specimens for different disease sites. The most common diagnostic tests on EPTB samples are:

- Smear for acid-fast bacilli (AFB);
- Liquid culture on fluids or tissue samples;
- Molecular (PCR) tests (e.g., Xpert MTB/RIF® by Cepheid);
- Histopathological examination of biopsy tissue;
- Adenosine deaminase (ADA) or free interferon-gamma levels in sterile fluids such as pleural, peritoneal and pericardial fluids.

Smears are often negative in EPTB specimens because of the low numbers of AFB. Liquid cultures and histopathology results are therefore critical. Molecular/PCR tests are helpful if positive. However, if PCR tests are negative, EPTB cannot be ruled out. This is because molecular tests for EPTB are highly specific, but sensitivity is not very high.[9-11] It is important to note that molecular tests for EPTB should not be performed on venous blood specimens. They should be used on specimens from the site of the disease.

SPECIMENS FOR CHILDHOOD TB

Table 2 provides a summary of various specimen collection methods for pediatric TB, and the perceived problems and/or benefits of each.[12] While older children may be able to cough up sputum samples, this is very difficult in young children since they tend to swallow sputum rather than expectorate them. In young children (<7-8 years of age), the routine specimens collected are two to three fasting gastric aspirates (gastric juice aspirate). However, the collection of 2-3 fasting, early morning gastric aspirate specimens is cumbersome and usually requires hospitalization. The following are basic guidelines for collecting gastric aspirates: 1) Specimens are collected after the child has fasted for eight to ten hours and, preferably, while the child is still in bed; 2) Specimens are usually collected daily for three days.

There is no adequate gold standard test for childhood TB, and diagnosis requires multiple tests.[13] Smears for AFB are often negative because of the low numbers of AFB in childhood TB. Therefore, liquid culture and molecular tests may be most helpful, along with signs, symptoms, chest radiology, evidence of TB infection (e.g., positive Mantoux skin test), and history of contact with active TB. The diagnosis and management of childhood TB will be covered in a future article in this series.

SPECIMENS FOR LATENT TB INFECTION (LTBI)

The diagnosis and management of latent TB infection will be covered in a future article in this series. Briefly, the goal of testing for latent TB infection is to identify individuals (e.g., close contacts of active TB cases) who are at increased risk for the development of active TB

Table 2 – Specimen collection methods for childhood TB[12]

Specimen collection method	Problems/Benefits	Potential clinical application
Sputum	Not feasible in very young children; Assistance and supervision may improve the quality of the specimen	Routine sample to be collected in children >7yrs of age (all children who can produce a good quality specimen)
Induced sputum	Increased yield compared to gastric aspirate; No age restriction; Specialized technique, which requires nebulization and suction facilities; Use outside hospital setting not studied; Potential transmission risk	To be considered in the hospital setting on an in- or out-patient basis
Gastric aspirate	Difficult and invasive procedure; Not easily performed on an outpatient basis; Requires prolonged fasting; Sample collection advised on 3 consecutive days	Routine sample to be collected in hospitalized patients who cannot produce a good quality sputum specimen
Nasopharyngeal aspiration	Less invasive than gastric aspirate; No fasting required; Comparable yield to gastric aspirate	To be considered in primary health care clinics or on an outpatient basis
String test	Less invasive than gastric aspirate; Tolerated well in children >4 years; Bacteriologic yield and feasibility requires further investigation	Potential to become the routine sample collected in children who can swallow the capsule, but cannot produce a good quality sputum specimen
Bronchoalveolar lavage	Extremely invasive	Only for use in patients who are intubated or who require diagnostic bronchoscopy
Urine/Stool	Not invasive; Excretion of *M. tuberculosis* well documented	To be considered with novel sensitive bacteriologic or antigen-based tests
Blood/Bone marrow	Good sample sources to consider in the case of probable disseminated TB	To be considered for the confirmation of probable disseminated TB in hospitalized patients
Cerebrospinal fluid (CSF)	Fairly invasive; bacteriologic yield low	To be considered if signs of tuberculous meningitis
Fine needle aspiration biopsy (FNAB)	Minimally invasive using a fine 23G needle; excellent bacteriologic yield,	Procedure of choice in children with superficial lymphadenopathy; minimal side-effects

Adapted from: Marais BJ. Pai M. Specimen collection methods in the diagnosis of childhood tuberculosis. *Indian J Med Microbiol* 2006;24:249-251.[12]

and therefore would benefit from treatment of latent TB infection (e.g., isoniazid for 6-9 months, after active TB is ruled out). Only those who would benefit from treatment should be tested, so a decision to test presupposes a decision to treat if the test is positive.

There are two accepted tests for identification of LTBI: the tuberculin skin test (TST) and the interferon gamma release assay (IGRA). As with the TST, IGRAs are surrogate markers of *Mycobacterium tuberculosis* infection and indicate a cellular immune response to *M. tuberculosis*.[14] In other words, both tests provide indirect evidence that the patient has been sensitized to *Mycobacterium tuberculosis* in the past. Neither test proves that the patient has current active TB disease, and should not be used to diagnose active TB.

IGRAs require blood samples, while the TST is an intra-dermal skin test (Mantoux technique). For IGRAs such as QuantiFERON-TB Gold®, blood must be collected in special antigen-coated tubes and shaken after blood collection to ensure that blood comes into contact with TB-specific antigens. Blood tubes are then incubated overnight and supernatants are then assayed via ELISA for interferon-gamma levels. It is important to strictly follow manufacturers' recommendations on IGRAs. Delays in incubating the blood can cause loss in sensitivity and increase the rate of indeterminate results.

TST should be performed using the Mantoux technique which consists of intradermal injection of tuberculin material (0.1 ml of purified protein derivative (2TU of PPD RT23)) on the inner surface of the forearm. A clear, raised wheal of 6-10 mm diameter should appear

CLINICAL HIGHLIGHTS

❑ A good diagnostic approach for tuberculosis (TB) requires collection of the right clinical specimen of good quality and quantity, and use of the appropriate laboratory test.

❑ All clinicians, therefore, should have basic knowledge about the types of specimens that can be collected, and should be able to provide clear instructions to their patients on how to provide such specimens.

❑ The specimen type is decided by the site of disease or purpose of testing or patient population.

❑ Sputum is the ideal specimen to collect for pulmonary TB. There are no validated, accepted blood-based tests for active TB.

❑ All patients suspected of having pulmonary TB should have at least two sputum specimens submitted.

❑ All patients should be instructed on the difference between sputum and saliva or nasopharyngeal secretions and the necessity for a deep, productive cough.

❑ Extrapulmonary TB (EPTB) can occur in many sites, the most common being lymph nodes, pleural, abdominal and meningeal sites. EPTB cannot be diagnosed with sputum or blood specimens. It is critical to make an effort to collect tissue and fluids from the site of the disease. This may require surgical expertise and referral to a center where biopsies can be done safely.

❑ Childhood TB can pose many challenges for specimen collection. While older children may be able to cough up sputum samples, this is very difficult in young children. In young children (<7-8 years of age), the routine specimens collected are two to three fasting gastric aspirates (gastric juice aspirate).

❑ There are two accepted tests for identification of latent TB infection (LTBI): the tuberculin skin test (TST) and the interferon gamma release assay (IGRA). IGRAs require blood samples, while the TST is an intra-dermal skin test (Mantoux technique).

when the PPD is slowly injected into the skin. The results should be read 48-72 hours after administration, by a trained professional. Transverse induration should be measured in mm. Redness (erythema) is not measured.An induration of 10 mm or more is usually considered positive for TB infection.

SOME COMMON SPECIMEN-RELATED ERRORS IN THE INDIAN CONTEXT

As mentioned previously, the most common error in the Indian context is use of blood (instead of sputum) as the specimen for active pulmonary and extra-pulmonary TB diagnosis. Indian labs not only perform blood tests like serology for TB, they also perform PCR tests on blood samples. These practices are unscientific and need to be discouraged. The exception would be use of blood culture or PCR for the diagnosis of disseminated TB in children or immune-suppressed persons. The more recent use of IGRAs like TB Gold for active TB is another cause for concern that will need to be addressed by clinicians and laboratory professionals in India. ■

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