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. 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. 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

<table>
<thead>
<tr>
<th>Site, purpose, or patient population</th>
<th>Specimen of choice</th>
<th>Comments</th>
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</table>
| **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**       | 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 |
| TB lymphadenitis                    | 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 |
| Pleural effusion (TB pleuritis)     | 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 |
| Ascites (abdominal TB)              | 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 |
| TB meningitis                       | 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 |
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 specimen-scan 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.
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.

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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:

- 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.
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 > 2 hours, 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 BacTAlert 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.
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, IGRA are surrogate markers of Mycobacterium tuberculosis infection and indicate a cellular immune response to M. tuberculosis. 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 IGRA 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 IGRA. 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

### Table 2 – Specimen collection methods for childhood TB

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

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.

**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). IGRA require blood samples, while the TST is an intra-dermal skin test (Mantoux technique).

**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 IGRA like TB Gold for active TB is another cause for concern that will need to be addressed by clinicians and laboratory professionals in India.

**REFERENCES:**

Questions

1. What is the ideal location for sputum collection?
   a. Inside the doctor's clinic
   b. Inside the laboratory
   c. Outdoors or in a well ventilated area
   d. The patient's bathroom with the door closed

2. Which of the following tests requires a blood sample for the diagnosis of active pulmonary TB?
   a. Sputum smear microscopy
   b. Liquid culture
   c. Interferon-gamma release assay (IGRA)
   d. None of the above

3. Which of the following constitutes a good sample for sputum microscopy?
   a. 5 ml of muco-purulent sputum
   b. 5 ml of clear saliva
   c. 2 ml of sputum in a washed food container
   d. All of the above

4. For a two year old child with suspected TB, the best clinical specimen for pulmonary TB diagnosis is:
   a. Blood
   b. Sputum
   c. Gastric aspirate
   d. Bronchoalveolar lavage (BAL)

5. What is the minimum recommended number and timing of specimens for the diagnosis of pulmonary TB?
   a. Three early morning sputum specimens
   b. Two sputum specimens collected one hour apart
   c. Three sputum specimens taken on the same day
   d. One early morning sputum plus one blood sample

6. Which of the following specimens should be rejected by the culture laboratory?
   a. An unlabeled sputum specimen
   b. A lymph node received in fixative
   c. A specimen received without a request form
   d. All of the above

7. In a woman with infertility, suspected to have genito-urinary TB, which of the following specimens is important for diagnosis?
   a. Venous blood
   b. Menstrual blood
   c. Endometrial tissue
   d. Sputum

(See answers on the next page)
Answers

1. The correct answer is (c). Sputum expectoration can release infectious aerosols, which are diluted in the open-air or a well ventilated room and are inactivated by UV light in the outdoors.

2. The correct answer is (d). There is no approved blood test for the diagnosis of active pulmonary TB. IGRAs are tested on blood but only give information on latent TB infection, not active disease.¹

3. The correct answer is (a). Sputum quality and quantity are essential features of a good specimen for TB testing. Five ml of muco-purulent specimen collected in a sterile laboratory approved container is the ideal specimen for sputum microscopy for TB.

4. The correct answer is (c). Young children cannot expectorate sputum but it is often swallowed overnight. The best specimen is, therefore, a series of two to three consecutive, fasting gastric aspirates.²

5. The correct answer is (b). The minimum requirement for sputum testing is two specimens taken one hour apart.³ Previously three consecutive early morning sputum samples were recommended but research has shown that the 3rd sample does not add much to the overall yield (and can increase the risk of patient drop-out).⁴

6. The correct answer is (d). Specimens that are unlabeled or do not have a properly filled out form, are rejected by the laboratory. Fixatives, such as formalin, will inactivate any micro-organisms in a tissue sample, therefore, making it unsuitable for culture.

7. The correct answer is (c). Endometrial issue for culture and histopathology is critical for diagnosing genito-urinary tract TB. Blood (venous or menstrual) is not the appropriate sample as it is very difficult to isolate M. tuberculosis or detect MTB DNA in such samples.

REFERENCES: