

LABORATORY DIAGNOSIS OF MYCOBACTERIAL INFECTIONS

Chapter

24

Consider tuberculosis (TB) as part of the differential diagnosis of selected patients, especially those with respiratory tract infections, lymphadenopathy, fever of unknown origin, night sweats and weight loss to avoid delays in the diagnosis and inappropriate treatment. Staining for acid-fast bacilli thus forms part of routine sputum microscopy, culture and sensitivity testing (MC&S). Specific laboratory tests for mycobacterial infection should be ordered, e.g. TB culture and TB polymerase chain reaction (PCR), if TB is clinically suspected.

Prompt diagnosis facilitates timely therapeutic intervention and minimises the risk of community transmission. It should be noted that establishing a definitive laboratory diagnosis of TB is not possible in 15–20% of patients with a clinical diagnosis of TB. In such cases, the presumptive clinical diagnosis is often sufficient for initiating anti-tuberculous therapy.

The rapid rise in drug-resistant TB necessitates tests that determine drug susceptibility to ensure initiation of effective treatment and to monitor drug susceptibility trends. Multi-drug-resistant TB (MDR-TB) and extensively-drug-resistant TB (XDR-TB) are both associated with a high fatality rate and require prolonged and alternative chemotherapy.

LABORATORY TESTS FOR MYCOBACTERIAL INFECTIONS

DIAGNOSING ACTIVE PULMONARY TB

Initiating a diagnostic evaluation of TB is usually based on suspicion of TB: clinical manifestations, a history of known/possible exposures, and radiological grounds.

SPECIMENS SHOULD BE SUBMITTED FOR:

- Acid-fast staining: sensitivity is ~ 45–65%
- Culture and sensitivity: 'gold standard' for laboratory confirmation of the TB diagnosis. Sensitivity is ~ 80–85%.
- TB PCR, e.g. GeneXpert TB PCR, allows for a more rapid laboratory diagnosis of TB. Sensitivity is ~ 70–75% for smear-negative pulmonary TB. The sensitivity of PCR is somewhat lower for non-respiratory specimens as compared to respiratory specimens.

CLINICAL SPECIMENS

- **Sputum (5–10 mL)**
 - **Expectorated sputum:** For patients that can voluntarily cough up sputum.
 - **Induced sputum (adults):** If a patient cannot voluntarily cough up sputum, an induced sputum specimen can be obtained by inhalation of aerosolised hypertonic saline generated by a nebuliser and it should be clearly marked 'induced sputum'. This procedure should be administered by qualified, trained personnel using personal protective equipment in

a controlled environment to minimise exposure to themselves and others. The results of induced sputum are comparable to bronchoalveolar lavage (BAL) but the procedure is safer and less costly compared to BAL.

- **Induced sputum (children):** In children, induced sputum has a higher yield than expectorated sputum. Induction is performed by aerosolisation of heated saline combined with salbuterol (or a similar agent to reduce wheezing). This is followed by suctioning to capture expectorated sputum. The procedure has been found to be safe and effective in children as young as one month of age.

At least three single specimens should be collected eight to 24 hours apart (with at least one specimen obtained early in the morning), although the diagnosis can often be made with two specimens. Early morning specimens before eating breakfast are preferred. Twenty-four hour collections of sputum are unacceptable because of the possibility of bacterial overgrowth.

- **Bronchoscopy with bronchoalveolar lavage** should be used only when attempts to obtain adequate expectorated or induced sputum have been unsuccessful, or sputum studies are negative in the setting of high clinical suspicion for pulmonary TB, or a potential alternative diagnosis for which bronchoscopy is required, exists.
- **Gastric aspirates** can be obtained in young children who are often unable to produce adequate sputum specimens and when sputum induction has been unsuccessful. Ideally three early morning specimens should be collected on different days before the child eats or ambulates to optimise the yield. The yield for mycobacteria by culture is generally low (30–40%) and even lower for acid-fast smears (< 10%). In addition, false-positive smear results may occur as a result of non-tuberculous mycobacteria present in the aspirate.
- **Nasopharyngeal aspiration** has been reported to have similar yields as gastric aspiration. It is less invasive and can be performed in the outpatient setting. Gastric aspirates are, in general, not used for adults.

DIAGNOSING EXTRAPULMONARY TB

In extrapulmonary TB, site-specific tissue or fluid specimens should be submitted for acid-fast staining, culture and sensitivity, PCR as well as histologic examination.

- **Fluids:** pleural, peritoneal and pericardial fluids may be collected for:
 - Routine studies, e.g. protein, glucose, LDH concentrations and these are compared to their corresponding serum concentrations; a cell count and differential count.
 - Microbiological studies e.g. acid-fast staining, culture and sensitivity, and PCR. Although a high protein (> 30 g/L), high LDH (\geq 200 U/L) and low glucose concentration together with a lymphocytosis are characteristic of tuberculous effusions, neither their presence nor absence are diagnostic. In addition, the organism burden in these fluids is relatively low, thus smears, cultures, and PCRs are often negative and hence indirect modalities such as adenosine deaminase (ADA) level, are often helpful. An elevated ADA level (> 30 IU/L) with compatible routine studies (including a cell count consisting of lymphocytes only) may be helpful to establish a presumptive diagnosis of TB effusion in the right clinical setting. It is important to remember that with ADA, non-tuberculous aetiologies may also be associated with elevated levels. An ADA result cannot be interpreted in isolation from the clinical picture and/or radiological and other laboratory features.
- **Cerebrospinal fluid (CSF):** A large volume of CSF (> 6 mL) should be submitted to the laboratory for optimal test sensitivity. Repeat lumbar puncture and CSF examination also increase the diagnostic yield. CSF should be submitted for protein, glucose, cell count and differential count, acid-fast staining, culture and sensitivity, and PCR. The protein is usually high, glucose low and a lymphocytosis present. Earlier in the course of illness the cellular reaction is atypical with only a few cells or with polymorphonuclear leukocyte predominance.

PCR can be helpful when positive but the overall sensitivity of PCR is too low to rule out TB meningitis. When TB meningitis is suspected, the importance of repeated sampling and careful examination cannot be overemphasised. Empiric therapy need not be delayed during the time of sampling.

- **Urine:** A minimum of 40 mL of midstream urine, at least three first morning specimens in suspected genitourinary tuberculosis should be collected. Twenty-four hour collections are not acceptable because of the likelihood of bacterial overgrowth. Submit for acid-fast staining, culture and sensitivity, and PCR.
- **Blood cultures** in specialised mycobacterial blood culture bottles may be collected in the setting of suspected disseminated infection. PCR is generally not performed directly on blood specimens due to its low sensitivity.
- **Pleural biopsy:** histological examination and culture of pleural tissue is the most sensitive investigation for TB pleurisy with caseating granulomas seen in 50–97% of cases of pleural TB. Pleural biopsy is warranted where there is a moderate to high suspicion of TB and pleural fluid evaluation is suggestive but not diagnostic of TB.
- **Lymph node:** fine needle aspiration (FNA) has a good yield for acid-fast bacilli. Aspirates should be submitted for acid-fast staining, culture and sensitivity, PCR as well as cytology. Excision biopsy for histological and microbiological evaluation has the highest diagnostic yield and should be pursued when FNA is not diagnostic. Excision biopsy specimens should be submitted for histology, acid-fast staining, culture and sensitivity, and PCR. Caseating granulomas on histology are highly suggestive of TB but not diagnostic. An incision biopsy is not recommended as it may complicate with sinus formation.
- **Other biopsy specimens** that may be sent for histology as well as acid-fast staining, culture and sensitivity and PCR include: bone marrow, lung, liver, epididymis etc. Specimens for histology should be collected in formalin. Specimens for molecular tests and TB culture should be collected in saline.

DIAGNOSTIC METHODS

MICROSCOPY FOR ACID-FAST BACILLI

Advantages: rapid and inexpensive

Disadvantages: lacks sensitivity:

- For a smear to be positive in a patient with pulmonary tuberculosis, there must be at least 10 000 acid-fast bacilli per mL of sputum.
- The Auramine O fluorescent stain is more sensitive than the Ziehl-Neelsen stain.
- The overall sensitivity of a single smear ranges between 45% and 65% in an immunocompetent person with pulmonary tuberculosis.
- Smear-negative pulmonary tuberculosis is particularly common in HIV-infected patients. Thus a negative smear does not rule out the diagnosis and cultures/PCR should always be performed due to the poor sensitivity of microscopy, the need for species identification and drug susceptibility testing.

TB CULTURE

ADVANTAGES

- Culture is the gold standard for the laboratory diagnosis of tuberculosis and can detect as few as 10–100 viable bacteria per mL of sputum.
- It is the most sensitive test and therefore the test of choice for diagnosing TB from extrapulmonary sites, such as CSF and effusions where the organism load is low.
- It detects not only *Mycobacterium tuberculosis* complex (MTBC) but the non-tuberculous mycobacteria (NTM) such as *M. avium-intracellulare* complex which may be clinically relevant. In addition, *Nocardia* infections are frequently detected in TB cultures which would otherwise have been missed.
- Full first- and second-line drug susceptibility can be done on positive cultures, which is important, as genotypic methods can miss resistant tuberculosis in certain circumstances and give indeterminate results on occasion which then require phenotypic-susceptibility testing.
- Culture detects viable bacteria and thus can be used to follow up patients on treatment to ensure that they are responding appropriately.

DISADVANTAGES

- Mycobacteria are slow growing and culture takes six to eight weeks on Lowenstein-Jensen solid agar slopes.
- The laboratory uses modern automated liquid-based culture systems (Mycobacteria Growth Incubator Tube (MGIT) and MB Bact/Alert) in which the time to detection is significantly reduced compared to older solid media culture methods. The mean time for detection of growth in liquid-based methods is reduced to seven to 21 days.
- Time to detection may be longer if there are few viable bacteria in the sample, hence samples that have not yielded growth are incubated for six weeks prior to a final result of no growth of mycobacteria being issued.

MOLECULAR METHODS (PCR) DIRECTLY ON THE SUBMITTED SAMPLE

ADVANTAGES

- PCR assays can detect approximately 100 bacilli per mL of sputum, making them less sensitive than culture but significantly more sensitive than microscopy.
- PCR testing can be done on almost any clinical sample and can be used for the diagnosis as well as for limited sensitivity testing of TB.
- There are also PCR tests that distinguish between MTBC and the NTM, or that can diagnose co-infection.

DISADVANTAGES

- Relatively high cost.
- Although they are almost 100% sensitive in smear-positive cases, the sensitivity of most commercial assays approaches 75% in smear-negative cases of TB. This means that while a positive result can usually be regarded as diagnostic of active TB, a negative result does not exclude the diagnosis and further tests such as culture are warranted.
- PCR tests detect DNA and not viable organisms, and thus cannot be used as a test of cure of TB following treatment, as the DNA can persist and be detected after successful TB therapy. Results must therefore be interpreted in the clinical context.

COMMERCIAL TB PCR TESTS USED IN AMPATH: ADVANTAGES AND LIMITATIONS

CEPHEID GENEXPERT PCR

DESCRIPTION	ADVANTAGES	DISADVANTAGE
<p>Detects: MTBC and rifampicin susceptibility</p> <p>Method: specimen is loaded directly into a processing cartridge</p> <p>Approximately 130 bacilli per mL of sputum is required to obtain a positive result</p> <p>Required specimen: respiratory tract specimens, lymph node aspirates, pus-like serosal fluid and CSF (provided sufficient volume is submitted)</p>	<ul style="list-style-type: none"> • Can be performed quicker than batch PCR tests • Detects rifampicin susceptibility 	<p>Does not detect isoniazid susceptibility. Hence, additional testing is required if positive</p>

SCREENING MYCOBACTERIUM TUBERCULOSIS COMPLEX PCR

DESCRIPTION	ADVANTAGES	DISADVANTAGE
<p>Detects : MTBC only</p> <p>Required specimen: can be performed on any type of specimen</p>	<ul style="list-style-type: none"> • Good rapid screening PCR for TB at least as sensitive as GeneXpert PCR • Can be performed on any type of specimen 	<p>Does not determine drug susceptibility. Positive results require further PCR-based drug susceptibility tests</p>

HAIN TB ASSAYS (VERSION 2.0)

DESCRIPTION	ADVANTAGES	DISADVANTAGE
<p>Detect: MTBC and drug susceptibility to first- and second-line anti-TB drugs</p> <p>Required specimen: direct clinical specimens e.g. sputum that are screening TB PCR positive as well as cultured TB isolates</p>	<ul style="list-style-type: none"> • Almost as sensitive as other commercial PCRs e.g. GeneXpert • Provide pharmacogenetic information which is useful to guide therapy e.g. INH resistance: <ul style="list-style-type: none"> katG promotor region mutation = high-level INH resistance inhA promotor region mutation = low-level INH resistance as well as predicting cross-resistance to ethionamide 	

MYCOBACTERIUM AVIUM COMPLEX PCR

DESCRIPTION	ADVANTAGES	DISADVANTAGES
Detects: <i>M. avium</i> and <i>M. intracellulare</i> species Required specimen: can be performed on any type of specimen	Useful test in HIV infected patients	Does not test for drug susceptibility



NOTE

To assess response to TB treatment, please request TB microscopy and culture and not PCR as molecular methods may detect persisting DNA from dead TB bacilli.

IDENTIFICATION OF POSITIVE ISOLATES (CULTURES)

The genus mycobacterium includes the MTBC (e.g. *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti*), also NTM (e.g. *M. avium*, *M. intracellulare*, and *M. kansasii*). These NTM can cause disease in certain groups, particularly immunocompromised patients such as in HIV-infected persons. Once growth is detected, species identification and drug susceptibility is performed using PCR-based methods (HAIN reverse-line probe assays) on the culture material. A line-probe assay is also available to distinguish the members of the MTBC, including *M. bovis* BCG and is useful in cases of suspected BCGosis.

DRUG-SUSCEPTIBILITY TESTING (DST)

Testing for TB drug susceptibility should ideally be performed in all patients with tuberculosis. If this is not possible, then DST should be carried out on patients who continue to have positive sputum AFB and/or cultures after two months of therapy, all health-care workers, prisoners, those with a history of previous TB and patients who have had contact with a known MDR-TB patient. Drug susceptibility to rifampicin and isoniazid (INH) are routinely tested by genotypic methods (PCR-based methods).

There are two types of DST

- Genotypic DST: molecular-based testing which detects only those mutations that are screened for.
- Phenotypic DST: culture-based testing which detects resistance irrespective of the underlying mechanism.

GENOTYPIC DST

The HAIN GenoType MTBDR_p/us PCR/line probe assay will detect the most common INH and rifampicin resistance conferring mutations found in MTBC.

- Rifampicin resistance: located in an 81-bp 'core region' of the rpoB gene (accounting for about 90% of high-level rifampicin resistance). The clinical relevance and management of low-level rifampicin resistance remains to be fully determined.
- INH resistance: located in several genes and regions, e.g. katG codon 315, inhA regulatory region and the aphC-oxyR intergenic region.

The HAIN GenoType assay for rifampicin and INH susceptibility detects mutations in the rpoB gene, katG codon 315 and inhA gene of MTBC. If resistance to rifampicin and/or INH is detected, additional second-line genotypic susceptibility tests including fluoroquinolones, cross-resistance

to aminoglycosides (amikacin and kanamycin) and cyclic peptides (capreomycin), and ethambutol are performed (HAIN GenoType MTBDRs/ PCR/line probe assay). Genotypic DST is followed by phenotypic DST confirmation on TB culture isolates.

ADVANTAGES

- Compared to conventional phenotypic DST, the sensitivity and specificity for detecting rifampicin resistance is 98% and 100%, and 95% and 100% for detecting INH resistance.

LIMITATIONS OF GENOTYPIC DST

- Mutations conferring phenotypic resistance are not all known, particularly for second-line TB drugs. As a result, molecular DST cannot replace phenotypic methods for all drugs as yet. The specificity of the current second-line assay (HAIN MTBDRs/) is > 98% for fluoroquinolones, aminoglycosides and cyclic peptides, and thus where resistance is detected it can be confidently regarded as resistant to that particular drug (a good rule-in test). However, the sensitivity of this assay for detecting drug resistance is often suboptimal and thus genotypic, second-line, drug-sensitive results do require confirmation by means of phenotypic methods. A new version (v. 2.0) of the HAIN MTBDRs/ is now available and AMPATH has validated this assay for routine use. The new version detects additional second-line drug resistance conferring mutations improving the assays ability to rapidly detect XDR-TB on both direct clinical specimens and positive TB cultures and will be implemented during 2017.
- Genotypic DST may not work if the amount of TB DNA in the specimen is below the limit of detection of the assay and thus not amplifiable. In this situation DST can only be performed on the positive TB culture. Please ensure that a TB culture is requested should a low positive TB PCR result be received from the laboratory to allow for confirmation of the result and appropriate DST.

PHENOTYPIC DST

LIMITATIONS OF PHENOTYPIC DST

- Although automated liquid-based DST (MGIT 960) permits rapid and accurate semi-quantitative resistance profiling of first- and second-line antituberculous drugs, this is performed at one critical concentration as opposed to testing susceptibility across a range of minimum inhibitory concentrations. The relative ratio of susceptible and resistance strains within the specimen will affect the result. Low-level resistance may be missed by phenotypic DST.
- Predicting cross-resistance between drugs within the same class can be difficult and depends on the drug used for screening and the critical concentration used.
- Liquid-based DST is subject to bacterial contamination and this problem may remain despite repeated decontamination procedures.
- Phenotypic DST for ethambutol and pyrazinamide is problematic and technically difficult.

USE OF THE URINARY LIPOARABINOMANNAN TEST

Testing for URINARY LIPOARABINOMANNAN (U-lam) with the Determine™ TB-LAM Ag test should be restricted to hospitalised HIV-infected adults with advanced immunosuppression ($CD4 \leq 100$ cells/mm³). All results should be interpreted in conjunction with the clinical and radiological picture of the patient.

ADVANTAGES OF THE U-LAM TEST

- It is a rapid, non-invasive and an inexpensive test.
- It offers an alternative diagnostic test in sputum-scarce HIV-infected patients.

DISADVANTAGES OF THE U-LAM TEST

- The Determine™ TB-LAM Ag test may only be used for urine samples; its use in other sample types remains to be determined.
- A negative U-lam test does not rule out the possibility of TB due to its low sensitivity, hence additional specimens should be sent for microscopy, culture and molecular testing (PCR).
- No TB drug-sensitivity results are generated with this test.
- False positive test results from bacterial contamination are possible (commensal flora, including actinobacteria as well as *Candida* spp.).

DIAGNOSING LATENT TB

In most individuals, TB infection is contained by host defences and the infection remains latent. However, in certain individuals, e.g. underlying immunosuppressive disease such as HIV infection, lymphoma, leukaemia, head and neck cancer, transplant patients, treatment with chemotherapy, renal failure requiring dialysis or other major immune-compromising conditions, latent infection has the potential to develop into active disease. Children under the age of five years are also at risk of developing active TB following latent infection. Normal healthy individuals with latent TB have an annual risk of 0.1% for developing active TB, compared to 10% in HIV-infected individuals.

The goal of testing for latent infection is to identify individuals who are at increased risk for developing active TB and therefore would benefit from latent TB treatment. Only those who would benefit from treatment should be tested. A decision to test presupposes treatment if the test is positive. Testing patients who have a low risk for TB is discouraged.

The treatment of latent TB (e.g. isoniazid daily for nine months) is very different from treating active TB which requires multidrug regimens.

There are two major tests (tuberculin skin test (TST), and interferon-gamma release assays (IGRAs)) for the identification of latent TB infection. They both measure the T-lymphocyte response to TB antigens. Neither can differentiate active from latent tuberculosis. The ability to detect those cases at highest risk to progress to active disease is poor. Reliable prediction of future disease amongst those with a positive result is not possible and strong positive test results do not suggest higher risk. In general, use of these tests is not recommended for diagnosing active TB in adults.

THE TUBERCULIN SKIN TEST

The tuberculin skin test (TST) is used to identify individuals with previous sensitisation to mycobacterial antigens. Currently, the only recommended TST is the Mantoux test. This test consists of intradermal injection of five tuberculin units (TU) of purified protein derivative (PPD) or two TU PPD RT23 (these are considered equivalent). A delayed-type hypersensitivity reaction will occur within 48 to 72 hours in a person who has cell-mediated immunity to these tuberculin antigens. The reaction will cause localised induration of the skin at the injection site, and the transverse diameter should be measured (as millimetres of induration) by a trained individual and interpreted using risk-stratified cut-offs. In asymptomatic persons, exposure to MTBC may be demonstrated by a positive skin test. Those with a strongly positive skin test are presumed to be infected with MTBC but it does not mean active disease. Patients with a positive TST must undergo clinical evaluation to rule out active TB and to assess the need for treatment.

LIMITATIONS OF THE TUBERCULIN SKIN TEST

- False positive results can occur because of the sensitising effect on the immune system of either prior BCG vaccination or opportunistic environmental mycobacteria (NTM). A positive Mantoux skin test caused by BCG or NTM can be distinguished from latent TB by the IGRAs.

- False-negative results can occur due to immunosuppression, particularly co-infection with HIV. Extensive TB (pulmonary or miliary) can also temporarily depress the immunity, and can lead to a false negative TST.
- The TST is also known to have problems with reproducibility, with inter- and intra-reader variability with the measurements of induration.

INTERFERON-GAMMA RELEASE ASSAYS

Interferon-gamma release assays (IGRAs) have been developed using the *Mycobacterium tuberculosis* antigens 'early secretion antigen target 6' (ESAT-6) and 'culture filtrate protein 10' (CFP-10), which are not present in BCG, and are found in only a few species of environmental mycobacteria (*M. marinum* or *M. kansasii*). These in-vitro blood tests assess a cell-mediated response in infected persons by determining the amount of interferon-gamma released when sensitised T-lymphocytes are stimulated by recognisable antigens ESAT-6 and CFP-10. These tests aim to be more specific by removing false positive results associated with BCG vaccination and infection with NTM. There is little data available supporting the use of these tests in those who are HIV positive and in children.

The two IGRAs that are available are:

- **T-SPOT-TB test (TB-Spot test)**
No specialised tubes are required for the T-Spot-TB test. The test requires two citrate (blue top) tubes.
- **QUANTIFERON-TB GOLD**
Special tubes are required for this test and specimens must be processed within 12 hours of collection. There are more indeterminate results with this test when compared to the T-Spot TB test.

A positive IGRA result is suggestive of previous TB exposure. The tests alone are not able to make a diagnosis of active TB infection. The interpretation of test results should be together with other laboratory tests, radiological findings and the clinical picture.



NOTE

All patients with a positive Mantoux skin test, T-Spot-TB assay or Quantiferon-TB gold assay should undergo a clinical evaluation to rule out active TB infection. This includes evaluation of symptoms (e.g. fever, cough, night sweats), physical examination, chest X-ray and those with evidence of active disease should have specimens submitted to the laboratory for acid-fast staining, culture and PCR.