



PHLN

Public Health Laboratory Network

Tuberculosis (*Mycobacterium tuberculosis*)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for *mycobacterium tuberculosis*.

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1 PHLN Summary Laboratory Definition

1.1 Condition:

Tuberculosis

1.1.1 Definitive Criteria

- (i) Isolation of *M. tuberculosis* from an appropriate specimen.
- (ii) Positive test for *M. tuberculosis* DNA in an appropriate specimen in which acid fast bacilli have been identified.

1.1.2 Suggestive Criteria

- (i) Detection of acid fast bacilli in an appropriate specimen from a patient with disease typical of tuberculosis.
- (ii) Positive test for *M. tuberculosis* DNA in appropriate specimen in which acid fast bacilli have NOT been identified

1.1.3 Comments

All specimens for mycobacterial diagnosis should be submitted for culture which is more sensitive than polymerase chain reaction (PCR) and provides an isolate for antibiotic susceptibility testing and molecular typing. However, the presence of *M. tuberculosis* DNA in fixed tissue, with histopathological changes consistent with tuberculosis, including acid fast bacilli, is acceptable as a definitive criterion for the diagnosis of tuberculosis if fresh specimens are not available for culture.

If the presence of *M. tuberculosis* DNA or acid fast bacilli is not subsequently confirmed by isolation of *M. tuberculosis*, from at least one suitable specimen, using appropriate laboratory methods, the original clinical and laboratory criteria for the diagnosis of tuberculosis should be reviewed in consultation with the treating clinician. The patient's response to antituberculous therapy may also help to confirm the diagnosis.

2 Introduction

Tuberculosis is caused by *M. tuberculosis* or other members of the *M. tuberculosis* complex (MTBC), *M. bovis* (uncommonly) or *M. africanum* (rarely and in limited geographic areas). The pathogenesis, clinical manifestations and difficulties associated with the diagnosis of tuberculosis are largely determined by the characteristics of the organism, which include its slow growth and replication, thick, waxy cell wall and resistance to environmental conditions, disinfection, intracellular killing and antibacterial agents.

Primary infection with *M. tuberculosis* is usually acquired by the respiratory route and typically is asymptomatic. Symptomatic primary infection occurs particularly in children and people who are even mildly immunocompromised and in a small proportion of apparently otherwise normal individuals. More commonly infection is inapparent and contained either locally, in the lung, or after haematogenous spread to other organs, by the host's cell-mediated immune response. Although bacterial replication is inhibited, viable organisms remain in tissues indefinitely and can reactivate, often many years later, when the immune response is impaired for any reason. The commonest manifestation of tuberculosis in industrialized countries, is reactivation of lung infection in the elderly leading to pulmonary cavitation, with cough and sputum production. However, primary tuberculosis and reactivation of disease can occur in any organ and at any age.

The diagnosis of tuberculosis depends on isolation of *M. tuberculosis* or detection of its nucleic acid in appropriate clinical specimens. For various reasons isolation of *M. tuberculosis* may be impossible, despite a positive acid fast stain or histological evidence of mycobacterial infection e.g. because the patient has commenced therapy before specimens have been collected or fixed tissue is the only specimen available. In most cases, the diagnosis can be confirmed by detection of specific DNA. This should be attempted if possible because nontuberculous mycobacteria can cause disease which is indistinguishable from tuberculosis but requires different therapy and public health response. If a case of tuberculosis has been notified on the basis of positive microscopy only, the notification should be updated when the results of culture and/or DNA testing are available.

Serology has no established role in the diagnosis of tuberculosis. Tests for cell-mediated immune (CMI) or delayed type hypersensitivity responses to antigens of the *M. tuberculosis* complex (e.g. tuberculin skin test, TST; Quantiferon® assays) cannot differentiate active tuberculosis disease from quiescent infection, and occasionally give false-negative results in patients with active TB disease. Hence, these tests can assist in diagnosing active TB disease in only very few circumstances (e.g. paediatric and extrapulmonary cases). In these settings, an experienced clinician must evaluate the TST or Quantiferon result in combination with the patient's history, clinical features and other test results. These CMI tests, which are really used to detect latent tuberculosis infection (LTBI), will not be discussed further in this laboratory case definition.

The National Tuberculosis Advisory Committee has recently published guidelines for mycobacteriology laboratories in Australia.¹ The reader is referred to the NTAC document for more detailed guidance on the performance of TB microscopy, culture, susceptibility testing and nucleic acid detection (NAD) testing.

3 Tests

3.1 Suitable Specimens

Mycobacterium tuberculosis has the potential to infect any part of the body. Specimens such as joints, pleural, ascitic and cerebrospinal fluid, blood and tissues are normally considered to be sterile. Others such as respiratory, genitourinary and skin may be contaminated by commensal organisms. Other pathogenic organisms may be present in pus and necrotic tissues. Specimens other than those from normally sterile sites are decontaminated prior to culture using acid or alkali for a set time to reduce the number of viable commensal organisms without greatly reducing the number and viability of mycobacteria present. Specimens containing mucus, typically of respiratory origin, require the addition of a mucolytic agent to liquefy the specimen to allow the decontaminant to reach all organisms. Specimens from superficial sites (e.g. skin biopsy) or lymph node need to be subcultured at 30 ° C as well as 35 ° C to detect temperature-sensitive nontuberculous mycobacteria (NTM) that also cause skin infections.

Specimens should be collected into clean, sterile containers and should reach the laboratory within two days of collection, if possible. However, *M. tuberculosis* is a hardy organism and can sometimes be recovered from specimens up to five days old. If delay is unavoidable, specimens should be refrigerated.

3.1.1 Respiratory specimens

Sputum is the most commonly received specimen. The most sensitive collection protocol providing the highest yield of positive results is to collect an early-morning sample on three separate days. However, the “spot-morning-spot” protocol (ie. collection of two random sputa and one early-morning sample) is almost as sensitive and more convenient. Reject if insufficient specimen, 24 hour collection or multiple specimens received on the same day. The presence of numerous squamous epithelial cells in a sputum specimen indicates a poor quality sample. These poor quality samples may be rejected if other high-quality specimens are available. If no high-quality specimens are available, the poor-quality specimen may be processed (as some will still be positive) but a comment should be added to the final report so that the treating doctor is aware that a negative result may be due to poor specimen collection.

Other respiratory specimens include bronchoalveolar lavage, pleural fluid and pleural biopsy (which is preferable to fluid only, from patients with pleural effusions). Although a bronchoscopically collected specimen is preferable, induced sputum is sometimes collected from patients with minimal cough. However, great care is required to prevent aerosol transmission of tuberculosis and collection should only be undertaken in an isolation room or cubicle with negative air pressure and by staff with suitable protection (gown, mask and gloves).

3.1.2 Gastric aspirate

These are useful for patients who are unable to produce sputum, especially young children. They should be collected in the early morning before eating. Three specimens up to 50ml each should be sent. These specimens have very low pH, which may affect the viability of mycobacteria with time and must be neutralised within 4 hours of collection. Microscopy results for gastric aspirates may be misleading owing to the presence of environmental mycobacteria in food and water.

3.1.3 Urines

Three daily early morning (whole voided) urine collections. Reject if multiple specimens collected on the same day, insufficient specimen (< 20ml), or a 24 hour collection.

3.1.4 Faeces

Not usually recommended as suitable for mycobacterial examination because of the low yield of positive findings and the difficulty of removing contaminants prior to culture. They are usually only performed on HIV positive or other immunocompromised patients with suspected gastrointestinal tuberculosis.

3.1.5 Cerebrospinal fluid

Tuberculous meningitis is uncommon in Australia. It is life-threatening and laboratory diagnosis is crucial, but often difficult. It is usually suspected when CSF examination shows a typical “aseptic” meningitis pattern – usually a predominant lymphocytosis often associated with very high protein (i.e. 1.0 – 5.0 mg/ml) and low or undetectable glucose levels - in a patient with progressive, subacute neurological symptoms. However, these typical changes are nonspecific and often absent early in the course of illness, when the response to therapy will be best.

Acid fast stain and CSF culture for *M. tuberculosis* are often negative because of the low number of bacteria present and the small volumes submitted. Increasing the volume of CSF cultured will increase the sensitivity. A total of 10-20 ml of CSF can be collected by repeated lumbar punctures, and the diagnostic yield is not compromised for at least two weeks by the institution of empiric antituberculous therapy.

3.1.6 Blood

In patients with primary tuberculosis or who are immunocompromised *M. tuberculosis*, may be cultured from blood. Blood should be collected directly into a suitable liquid medium designed for mycobacterial culture, using aseptic technique and according to the manufacturer’s instructions.

3.1.7 Other specimens

Rejected if specimen in formalin, or on a dried swab.

3.2 Direct Microscopy

Microscopy for acid fast bacilli (AFB) may be performed on a smear made from the specimen prior to or following decontamination and concentration of the specimen. Stains used are Ziehl Neelsen and fluorochrome stains (most commonly auramine O-phenol).

3.2.1 Suitable specimens

All specimens except gastric aspirate and urine which may give false positives due to presence of environmental contaminating AFB. However, many laboratories do report results of urine microscopy believing that the presence of environmental AFB is overstated.

3.2.2 Test sensitivity

Requires at least 10,000 organisms / ml specimen to be reliably smear positive. Fluorochrome methods are more sensitive because smears can be scanned under 25X objective rather than 100X for ZN. All microscopy methods are insensitive compared with culture and nucleic acid detection methods (approximately 60% of culture positive specimens are positive by microscopy).²

3.2.3 Test specificity

These stains are moderately specific for mycobacteria; a few aerobic actinomycetes are also acid fast (but usually weakly). Microscopy cannot discriminate *M. tuberculosis* from other mycobacteria or viable from non-viable organisms. Newly positive fluorochrome smears should be overstained with ZN to confirm presence of AFB

3.2.4 Predictive values

Negative microscopy does not exclude a diagnosis of TB. Positive microscopy usually indicates the presence of mycobacteria, but false positive results due to contamination of slides or stains occasionally occur.

3.2.5 Suitable test acceptance criteria

Pale-robust staining AFB, may be quite pleomorphic and may display beading and/or banding. Need to identify greater than 3 AFB in the smear before reporting a positive microscopy result. An internationally recognised quantitation scheme is available in most textbooks. A smear positive for AFB requires immediate notification to the requesting Medical Officer by telephone and reporting to the state Tuberculosis Services Clinic.

3.2.6 Suitable internal controls

Smear positive (*M. bovis* BCG) and negative control smears used to check each new batch of reagents and each staining run.

3.2.7 Suitable validation criteria

A positive culture usually validates the results. Failure to isolate *M. tuberculosis* from a smear-positive specimen may be due to the presence of nonviable organisms in a treated patient with tuberculosis. If the specimen is not from a patient with known tuberculosis, the smear should be reviewed and the possibility of contamination or a false positive reading considered. Smear positive specimen concentrates should be retained for confirmatory nucleic acid detection if culture is negative in a patient in whom tuberculosis is strongly suspected.

3.2.8 Suitable external QC programme

NATA/RCPA

3.3 Culture

3.3.1 Media

Culture media used for isolation of mycobacteria must provide a variety of complex nutrients, including lipids and, for culture of specimens with normal flora, antibiotics or disinfectants to inhibit overgrowth during prolonged incubation. The traditional solid medium, Lowenstein-Jensen, contains egg yolk as

the main nutrient source and malachite green as disinfectant. Mycobacteria grow significantly more rapidly in liquid media, a variety of which, with different growth and antibiotic supplements and semi-automated or automated detection systems, are commercially available. The choice of media depends on the preference of the laboratory, most of which use a combination of both liquid and solid media

3.3.2 Test sensitivity

Culture is the 'gold standard' for detection of mycobacteria in clinical specimens and the sensitivity is very high (>95% of specimens containing viable bacteria) if both liquid and solid media are used and three separate specimens are cultured. The sensitivity of sputum culture is related to the efficiency of the decontamination process plus the quality, volume and adequacy of the specimen submitted. The lower limit of detection is 10 –100 viable organisms.

3.3.3 Test specificity

Other acid fast bacteria grow in the same culture media and must be differentiated from MTBC.

3.3.4 Predictive values

Negative: A negative culture does not exclude the diagnosis of TB.

Positive: Molecular typing has demonstrated a significant rate of culture contamination. Cultures can become contaminated with *M. tuberculosis* from strongly positive specimens during specimen processing, inoculation of media or in liquid culture systems that require invasive sampling of culture headspace gas to detect growth. Contamination should be suspected when *M. tuberculosis* is isolated, unexpectedly, from a specimen that was negative for acid fast bacilli. It can usually be confirmed by comparison with other isolates identified in the laboratory at about the same time by molecular typing.

3.3.5 Suitable test acceptance criteria

Acid fast bacillus (long, slender, gently curving bacillus with eccentrically located beading). Slowly growing on Lowenstein-Jensen or similar media producing rough, dry, buff-coloured colonies. Ziehl Neelsen (ZN) stained cultures (from liquid or solid media) usually demonstrate bacilli in a 'cording or serpentine' arrangement. Preliminary identification test yields a positive result for MTBC.

3.3.6 Suitable internal controls

Need to ensure media are capable of supporting the growth of MTBC by inoculating samples with standard strain of *M. tuberculosis* and *M. bovis*. Preliminary identification tests use positive and negative controls for each run

3.3.7 Suitable validation criteria

Culture is the 'gold standard' for detection of MTBC

3.3.8 Suitable external QC programme(s)

NATA/RCPA. The American College of Pathologists runs an excellent program but it is extremely difficult to obtain in Australia. The Australian Mycobacterial Reference Laboratory Network runs a program annually amongst reference labs in Australia and New Zealand.

3.3.9 Special considerations

Multidrug resistant strains of *M. tuberculosis* (MDRTB) are present in low numbers in Australia at present. Any specimen known to contain, or considered likely to contain MDRTB should be referred to a PC3 facility for evaluation.

Well established reference laboratories are present in most states. They have the capability to perform nucleic acid detection (NAD) tests, specialised cultures, susceptibility testing, and most are able to perform epidemiological profiling.

Isolation of *M. tuberculosis* is associated with a risk of laboratory acquired infection; a high standard of laboratory hygiene is required and only experienced appropriately trained staff should be employed in the Mycobacterium laboratory. Specimens should be processed in a biohazard cabinet and positive cultures with an appropriate level of containment.

3.4 Identification of *Mycobacterium tuberculosis*

The Gen-Probe Accuprobe chemiluminescent-labelled DNA probe for MTBC and in-house PCR-based tests are the most commonly used techniques for the preliminary identification of MTBC.

Specific identification of *M. tuberculosis* is usually performed by reference laboratories and has implications in terms of treatment, patient isolation for those with smear positive respiratory secretions and public health investigations.

Tests used to differentiate *M. tuberculosis* from other members of MTBC, *M. bovis*, *M. africanum*, *M. microti* include: rate of growth on subculture; TCH sensitivity; niacin production; nitrate production; pyrazinamidase production; microscopic/macrosopic evidence of cording. *M. bovis* grows better on pyruvate- than glycerol-containing media. *M. bovis* BCG can be differentiated from other members of MTBC by HPLC. Several reference laboratories have PCR tests based on “regions of difference”, which can be used to discriminate members of the MTBC.³

3.5 Subtyping of *M. tuberculosis*

Several methods including various PCR-based methods, Southern hybridisation using the IS 6110 gene as probe (restriction fragment length polymorphism, RFLP) and spoligotyping are commonly used to type *M. tuberculosis*. Of these, Southern hybridisation with IS 6110 is the most discriminatory and internationally recognised as the “Gold Standard” with RFLP based on polymorphic G-C-rich repetitive sequences (PGRS) as back-up. The disadvantages of IS 6110-based RFLP are that: the insertion sequence is not found in all isolates; the method requires a large amount of DNA and hence prolonged culture; and the method is technically demanding and time-consuming.

PCR-based methods, particularly mycobacterial interspersed repeat units (MIRU) typing, have therefore become the preferred TB typing techniques because small quantities of low quality DNA can be tested in simple PCR reactions. MIRU genotyping categorizes the number and size of repeat elements in each of 12-20 independent loci, using PCR followed by gel electrophoresis. The PCR methods are useful for rapidly determining whether MTBC isolated from consecutively processed specimens are likely to be real or due to a contamination event. They can also be used as a screening method to identify transmission or clusters of tuberculosis.

3.6 Direct Detection using Nucleic Acid Based Tests

Nucleic acid detection (NAD) tests, which are more sensitive and specific than microscopy and but less sensitive than culture are now available commercially. At least three formats of amplification are commercially available: Roche, polymerase chain reaction (PCR); Artus real-time PCR, and Gen Probe, isothermic amplification. In-house assays have also been developed by several laboratories and the comments below are also applicable to them. The use of these tests has significantly improved the diagnosis of tuberculosis by reducing the time for confirmation of suspected disease but they should always be used to complement (not replace) culture.

3.6.1 Suitable specimens

According to the manufacturers' guidelines. NAD assays either have FDA approval or are validated only for respiratory specimens, which have been concentrated, decontaminated and digested using either the NaOH or NALC/NaOH protocols. However, a number of published reports and increasing experience suggest that *M. tuberculosis* can be detected reliably in other specimens using these methods.

3.6.2 Test sensitivity

See manufacturer's kit inserts for values. In general, commercially available NAD assays have a high sensitivity (>95%) in ZN-positive specimens, but variable sensitivity, compared with culture (as low as 17 %) in ZN-negative specimens. A negative result does not exclude the diagnosis of TB.

3.6.3 Test specificity

See kit insert - generally highly specific. Most commercial NAD kits are formatted to reduce the risk of contamination, which is uncommon if appropriate precautions are taken. However, contamination can occur in any NAD test. Ideally a separate, unopened specimen should be submitted for NAD testing, as contamination of the specimen during processing can result in a false positive NAD test.

3.6.4 Predictive values

See kit insert. Positive predictive value depends on the choice of specimen and the prevalence of tuberculosis in the population. In a low prevalence community NAD tests for tuberculosis should not be done unless there is either a positive ZN smear or there is a very high clinical suspicion of tuberculosis. (i.e. they should not be done routinely on all specimens submitted for mycobacterial culture). NAD tests cannot be used to monitor treatment.

3.6.5 Suitable test acceptance criteria

Positive or negative result accepted provided the kit's internal controls and the laboratory positive and negative controls have produced expected results, and the inhibition control is negative. However, a positive result that is not subsequently confirmed by culture should be reviewed, unless the patient is known to have tuberculosis.

3.6.6 Suitable internal controls

Positive (*M. tuberculosis* or *M. bovis* BCG) and negative control (*M. goodii* or similar) at appropriate dilution, blank (no DNA) controls where appropriate and an inhibition control check for each specimen.

3.6.7 Suitable validation criteria

Culture is the gold standard

3.6.8 Suitable external QC programme

Limited availability

3.6.9 Special Considerations

NAD tests should not be the sole test used for the diagnosis of TB. All specimens should be cultured for *M. tuberculosis* because of the superior sensitivity of culture, the need to confirm identity of the isolate, perform sensitivity tests and subtype which is useful not only for epidemiological purposes but to exclude the possibility of contamination by a previously processed positive specimen. Since these NAD tests are expensive laboratories may have criteria for selection of specimens to be tested. These may include pressing necessity for identification of cases for public health reasons and for immunocompromised patients where delay in diagnosis may adversely affect the patient's prognosis.

Kit inserts should be consulted for details of sensitivity and specificity of individual tests.

3.7 Susceptibility testing

3.7.1 Methods

Susceptibility testing of *M. tuberculosis* is usually performed by a reference laboratory. All initial isolates of *M. tuberculosis* from individual patients should be tested for susceptibility to at least the first line antituberculosis drugs, namely isoniazid, rifampicin and ethambutol. Further isolates from the same patient should be tested if the response to therapy is delayed or there is evidence of relapse of disease after treatment. If the isolate is resistant to any first line drugs, it should be tested for susceptibility to second line agents, after consultation with the treating physician.

Most laboratories now use the BACTEC radiometric or the non-radiometric MGIT systems to compare growth of the test organism in broth containing a single specified concentration of the antibiotic with its growth in antibiotic free medium. The method is qualitative and uses a single "breakpoint" antibiotic concentration for isoniazid, rifampicin, ethambutol, streptomycin, (although this agent is now rarely used for treatment) and for several of the second line drugs. Results are reported as susceptible or resistant. It is relatively rapid (read after 7-10 day's incubation), the results correlate well with the solid-media-based methods and, for *M. tuberculosis*, with response to therapy. Quantitative testing is sometimes requested e.g. to identify isolates with intermediate resistance to isoniazid which may be effective when combined with several other drugs to which the isolate is susceptible.

Pyrazinamide susceptibility is complicated by the fact that it is active only at a low pH at which *M. tuberculosis* grows poorly on solid media. Susceptibility can be measured using the radiometric method with adjustment of the pH. However, for *M. tuberculosis* (but not necessarily other Mycobacterium species), susceptibility to pyrazinamide generally correlates with activity of the enzyme pyrazinamidase (which converts pyrazinamide to pyrazinoic acid). Therefore, many laboratories use a positive pyrazinamidase test, which is one of the standard confirmatory tests for identification of *M. tuberculosis* to infer susceptibility to pyrazinamide. *M. bovis* and a small proportion of strains of *M. tuberculosis* fail to produce this enzyme and are intrinsically resistant to this agent.

4 References

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