

Guideline:

Laboratory Diagnostic Tests and Interpretation

Introduction

The purpose of this section is to outline the main methods of laboratory tuberculosis (TB) confirmation available to clinicians in New South Wales (NSW). In NSW all mycobacteria recovered from clinical samples are forwarded to the NSW Mycobacterium Reference Laboratory (MRL) at ICPMR-Pathology West for full identification, susceptibility testing, genotyping and more recently whole genome sequencing (WGS). This practice ensures compliance with NSW TB Control Program requirements about the timeliness and quality of identification of *Mycobacterium tuberculosis*, as well as compliance with laboratory safety regulations. It also ensures detection of drug resistant strains with ongoing surveillance of potential local transmission, in order to guide public health responses.

Clinicians ordering the TB tests and laboratories referring isolates for confirmatory testing should always provide basic patient information, including country of birth, relevant clinical history and date of specimen collection. Additional information regarding related laboratory test results (i.e. direct smear, PCR or TB antigen testing) is also useful.

Laboratory Biosafety Practices

Pathology providers should comply with the Australian/New Zealand Standard 2243.3 Safety in laboratories – Microbiological aspects and containment facilities, and with the National Pathology Accreditation Advisory Council (NPAAC) requirements. Staff working in any laboratory processing specimens for mycobacteria must wear protective laboratory clothing such as a solid-front or wrap-around gown, gloves and protective respiratory devices. Caution should be taken when performing aerosol-generating procedures such as centrifugation, vortexing, mixing, pipetting, pouring, and inoculation of culture media.

Pathology service providers should provide training programmes for biosafety on an annual basis to all staff members, to optimize compliance with local laboratory infection control protocols. New staff members should be certified before being allowed to work with potentially infectious TB specimens; all incidents of potential laboratory cross-contamination should be investigated and discussed with the treating clinicians. Multi-drug resistant *M. tuberculosis* is a Class III organism and must be handled in a Physical Containment Level III (PC3) laboratory. The NSW Mycobacterium Reference Laboratory has an accredited PC3 facility for this work.

Biosafety cabinets and all exposed work surfaces should be disinfected with bleach (hypochlorite) or phenol-based disinfectant before and after every procedure. Diluted (working) bleach solutions must be prepared daily and stay at or above 0.5% chlorine – undiluted commercial bleach is usually 4-5%. Similarly, phenol-based disinfectants must be diluted daily, preferably with deionized (not “hard”) water, to 2-5%. Both types of disinfectants are only effective if left in contact with the contaminated material for at least 15 minutes. All waste containing mycobacteria should be placed in leak-proof containers or autoclavable plastic bags that contain disinfectant solution which can be sealed before being removed from the cabinet and autoclaved.

Specimens for microbiological diagnosis

Healthcare workers must follow strict respiratory infection control guidelines (1) and take into account the risk of exposure to TB when collecting respiratory samples. The pathology provider should be notified about referral of samples collected from patients with suspected MDR-TB.

Sputum

The patient should be positively identified and appropriate labels placed on allocated containers. The nature of the desired specimen and the sputum collection procedure should be explained to the patient. Sputum collection should occur in a well-ventilated environment (preferably outside) and never in the laboratory.

Sputum specimens must be at least 2-3 mL in volume and of adequate quality. Saliva or nasal discharge samples are not suitable. If the sample appears clear and water-like, without particles or streaks of mucous, the patient should be encouraged to submit a new specimen. Specimens should be sent to the laboratory in tightly capped, properly labelled containers. Upon arrival in the laboratory, the quality of sputum samples should be assessed. Specimens in broken or leaking containers should be discarded and another specimen requested. Blood-streaked sputum is suitable, but pure blood should not be examined.

Induced sputum

If a patient is unable to spontaneously expectorate an attempt should be made to induce sputum production using nebulized hypertonic saline. This might generate infectious aerosols and should be performed in a dedicated sputum induction room, or a negative pressure respiratory isolation room. The patient should be left in the room until coughing subsides, and the room should not be used until adequate air-exchanges have occurred. Refer to the NSW TB Program *Sputum Induction Guidelines* for detailed procedural information (2).

Bronchial alveolar lavage (BAL)

Other respiratory tract specimens that can be submitted for mycobacterial culture are bronchial secretions (minimum volume 2-5 mL) and bronchial alveolar lavage (BAL) specimens (minimum volume 20-50 mL). BAL is an invasive technique, but useful in the presence of focal disease or poor sputum production (despite use of induced sputum). It is advisable that a microbiologist or registrar in the laboratory be contacted beforehand to ensure selection of appropriate laboratory testing and timely reporting of findings back to treating clinicians.

Respiratory samples in children

In children less than eight years of age who are unable to expectorate, early-morning fasting gastric aspiration/lavage is the most common specimen collected. Induced sputum is also feasible, can be performed in all age groups and has a slightly higher mycobacterial yield. Gastric aspirates should be immediately transported to the laboratory and the stomach acid neutralised by adding 100mg of sodium bicarbonate.

Tissues and body fluids

Aseptically collected tissues should be placed in sterile containers without fixatives or preservatives and transported to the laboratory as soon as possible.

For the diagnosis of tuberculous lymphadenitis, fine-needle aspiration biopsy or lymph node excision (not incision) biopsy are preferred. A fine-needle aspiration biopsy should be taken using a fine 22-24 gauge needle; smears should be prepared “on the spot” and the needle rinsed into a mycobacterial culture bottle. It is important to inform both the pathologist and the microbiologist beforehand.

Under no circumstances should the material for microbiological examination be placed in formalin (used for the histopathological specimen) as it will kill any TB bacteria present.

Body fluids (spinal, pleural, pericardial, synovial, ascitic fluid, blood, pus and bone marrow) should be aseptically collected in sterile containers. Pleural effusion generally has a low mycobacterial yield, but this may be increased by collecting large fluid volumes (20-50mls) or performing direct bed-side inoculation. A pleural biopsy is a better specimen to collect.

Urine is expected to be contaminated with perineal flora. To minimise excessive contamination of urine specimens, external genitalia should be washed before specimen collection. Once received in the laboratory, a urine sample must either be processed immediately or centrifuged and the pellet refrigerated. As excretion of tubercle bacilli is intermittent, three consecutive early-morning midstream specimens must be collected.

Cerebrospinal fluid (CSF)

CSF is usually collected by lumbar puncture, during intraventricular shunt placement or by tapping a shunt reservoir. The CSF should be collected into a series of small sterile tubes (usually three). Tube 1 represents first collection and is used for biochemistry, and tubes 2 and 3 for microbiology. All tubes should be sent to Microbiology initially. At least 3mL is required for routine microbiology (cell count, Gram stain, routine culture) and an additional 0.5mL is required for each additional test. If tuberculous meningitis is suspected at least 10 mL of CSF should be sent for microscopy, culture and polymerase chain reaction (PCR) testing (the likelihood of confirming the diagnosis is directly proportional to the volume examined); preferably before any antimycobacterial treatment is started.

Blood or bone marrow culture

Special mycobacterial growth media (e.g., BACTEC Mycolytic F medium) is required to grow *M. tuberculosis* from blood or bone marrow specimens in patients with suspected disseminated/miliary

TB. These bottles can be obtained on request from microbiology laboratories and should be promptly returned to the laboratory after culture collection.

Specimen handling and transportation

Specimens can be refrigerated at 2-8°C until ready for transport to the laboratory. Refrigeration reduces the growth of contaminants in the specimen. If a refrigerator is not available, specimens can be held in coolers with ice packs. This does not apply to whole blood specimens, which should not be refrigerated.

Specimens should be delivered to the laboratory as soon as possible after collection, preferably within 1-2 hours (but no longer than 24 hours after collection where this is not possible). CSF must be processed by the laboratory within two hours of collection, otherwise the cell count may be falsely low due to cell lysis.

Methods of diagnosis

Direct microscopy for detection of mycobacteria

The purpose of AFB microscopy is to detect acid-fast bacilli (AFB) by microscopic examination of clinical specimens and cultures. All mycobacteria are acid fast, but other genera, such as *Nocardia* and *Corynebacteria* may be partially acid fast as well. Microscopy cannot be used to determine individual species, or confirm the presence of *M. tuberculosis*. The limit of detection of AFB microscopy is approximately 100 bacilli per mL.

Smears are prepared from tissues, decontaminated respiratory specimens and concentrated body fluid specimens. These smears are stained with fluorescent stains, either auramine O or auramine/rhodamine, or with Ziehl-Neelsen (ZN) method. A semi-quantitative grading system is used to report the number of AFB observed in stained smears. Both live and dead bacilli are stained and counted. The commonly used scoring systems are published by the World Health Organization (WHO) and the International Union against TB and Lung Disease (IUATLD) (3).

At least two sputum samples collected separately are recommended for patients with suspected pulmonary TB. Laboratory analysis of two consecutive sputum smear samples improves the diagnostic yield (compared to one sample only), and reduces time to diagnosis and accelerates treatment initiation (compared to 3 samples required in the past). Early morning specimens have the highest yield, but good diagnostic specimens can be collected at any time of the day – if quality criteria are met. It is not recommended to perform smear microscopy on very bloody samples due to poor sensitivity. It is also not recommended to routinely perform smear microscopy from urine samples due to the frequent detection of saprophytic mycobacteria colonising the urogenital tract.

Table 1: Grading Scale for AFB observed by Ziehl-Neelsen Stain

Microscopy findings (100 high power fields (HPFs) examined)	Reporting and scoring*
No acid fast bacilli seen	Negative. No AFB observed
1-9 AFB per 100 HPFs	Confirmation required**
10-99 AFB per 100 HPFs	Scanty (+)
1-10 AFB per field in at least 50 HPFs	Moderate (++)
More than 10 AFB per field in at least 20 HPFs	Numerous (+++)

*The number of AFB indicates how infectious the patient is. It is important to record exactly what you see.

** Confirmation required by another technician or repeat of AFB microscopy on another smear.

Detection of mycobacteria by nucleic acid amplification tests

Several in-house and commercial nucleic acid amplification tests are available in major laboratories across NSW. The majority of them have been validated and offer higher sensitivity than AFB microscopy (Table 1). These assays perform better on samples (e.g., sputum) which are smear-positive. At least 0.5 mL of specimen is required for Mycobacterium PCR (testing by GeneXpert requires 1mL of fluid). CSF supernatant is not suitable for mycobacterial PCR testing.

Table 2: Comparison of pathogen detection methods for mycobacteria

Method	Detection limit	Turn-around-time
AFB smear microscopy	10,000 AFB/mL	24-48 hours
Nucleic acid amplification test (NAAT or PCR)	10-100 AFB/mL	3-4 hours for real-time PCR, 2 hours by GeneXpert (24-72 hours for batched tests)
AFB culture	1-10 AFB/mL	90% of smear positive samples become culture positive within 10-14 days

Culture-based identification of mycobacteria

Mycobacteria are fastidious microorganisms and are cultured in a nutrient-rich medium, while contaminating bacteria are inhibited by the addition of alkali and a cocktail of antibiotics. Cultures are incubated in the laboratory up to 6 weeks but the majority of isolates grow within 2-3 weeks after inoculation. Positive cultures can be presumptively identified as *M. tuberculosis* complex or non-tuberculous mycobacteria and should be forwarded to the NSW Mycobacterium Reference Laboratory for confirmatory identification, DST and genotyping within 48 hours of culture positivity. Differentiation between *M. tuberculosis* and non-tuberculous mycobacteria has been often done by detection of MPT64 protein from culture.

Drug susceptibility testing (DST)

Major objectives for drug susceptibility testing (DST) are to ensure effective personalised treatment of cases and drug resistance surveillance. MDRTB is caused by strains resistant to at least isoniazid and rifampicin. DST is crucial for the management of MDR-TB and for preventing emergence of additional drug resistance. Drug resistance rates can be higher in patients with history of TB treatment. DST should be repeated for a definite case if the patient remains culture positive after three months of treatment. All patients with sputum smear positive disease should receive a genotypic test to exclude likely drug resistance, within 3 days of initial sputum collection. All positive culture and DST results affecting patient management should be phoned and faxed to the treating doctor and relevant TB Chest Clinic as soon as the results are available.

Detection of markers of drug resistance using nucleic acid amplification

For patients at risk for multi-drug resistant TB (MDRTB) AFB smear positive respiratory specimens can be tested for the presence of molecular markers of resistance to rifampicin by commercial (e.g., GeneXpertTB/RIF (Cepheid)) or in-house PCR assays. Identification of mutations in the rifampicin-resistance defining region of the *rpoB* gene is interpreted as a proxy for MDRTB. These PCR are licensed for smear-positive sputums only and the testing requests and positive results should be discussed with a microbiologist.

NSW TB Control Program recommends PCR-based detection of molecular markers of resistance to rifampicin (as the proxy for MDRTB) on smear-positive respiratory specimens in order to minimize the risk of delayed diagnosis of potentially highly infectious cases of drug-resistant disease. It can be done on one of the initial samples using GeneXpert or other rapid MDRTB detection technology.

Culture-based DST using phenotypic assays

All first-line drugs (isoniazid, rifampicin, ethambutol and pyrazinamide) are tested initially and second-line and third-line drugs are tested for MDRTB isolates in consultation between treating clinicians and the NSW MRL. For MDRTB cases injectable drugs (such as capreomycin and amikacin), the fluoroquinolones (ciprofloxacin and moxifloxacin), rifabutin, ethionamide or prothionamide, cycloserine, linezolid, clofazimine and PAS are tested. One drug can be tested as a representative of a family of drugs (e.g. rifampicin results can be extrapolated to rifapentine, prothionamide to ethionamide and vice-versa), or drugs from the same class can be tested individually as rifabutin susceptibility cannot be inferred from rifampicin and amikacin/capreomycin susceptibility does not correlate with streptomycin results. Assays and critical concentration cut-offs for the new drugs, bedaquiline and delamanid, are under development.

Genotypic DST using molecular markers of drug resistance

Phenotypic resistance results can now be complemented by molecular detection of mutations conferring resistance. If a mutation known to confer resistance (e.g. *rpoB* mutation leading to S531L substitution for rifampicin resistance) is documented, this confirms resistance. Conversely, if no mutation is observed in the genes known to confer resistance, this suggests that the strain is likely to respond to standard doses of antituberculous drugs.

Genotypic resistance can be assessed by whole genome sequencing of *M. tuberculosis* or application of PCR-based assays targeting individual genes. For example, the GenoType MTBDRplus assay (HAIN LifeScience GmbH) allows for the identification of *M. tuberculosis* complex and simultaneous detection of mutations in the *rpoB* and *katG/inhA* (high/low isoniazid resistance, respectively) genes, associated with in vitro resistance to isoniazid and rifampicin. The GenoType MTBDRsl simultaneously identifies *M. tuberculosis* complex and its resistance to fluoroquinolones (e.g., ciprofloxacin and moxifloxacin) and/or aminoglycosides/cyclic peptides (injectable antibiotics as capreomycin/amikacin) and/or ethambutol.

Culture-based characterisation of *Mycobacterium tuberculosis*

The NSW MRL has been prospectively genotyping all *M. tuberculosis* isolates recovered from patients in NSW. This molecular typing supports reconstruction of transmission pathways and outbreak investigations, differentiates between reinfection and reactivation, helps to identify risk factors for local transmission and laboratory cross-contamination events.

In the last two decades, multiple molecular typing methods for *M. tuberculosis* have emerged, with different levels of reproducibility, discriminative power and demands on technical expertise. The most widely applied typing methods are spoligotyping, MIRU-VNTR (Mycobacterial Interspersed Repetitive Units – Variable Number of Tandem Repeats) typing and IS6110 RFLP (Restriction Fragment Length Polymorphism) typing. Both RFLP and 24-loci-MIRU (or MIRU-24) typing have a high level of discrimination and reproducibility and can be used for strain typing. The result of MIRU-24 typing is a numerical code of usually 24 numbers each representing the number of tandem repeats at different loci. More recently, whole genome sequencing (WGS) based genotyping has emerged as the most high-resolution tool for *M. tuberculosis* characterisation.

The NSW MRL offers MIRU-24 and WGS genotyping for public health laboratory surveillance in coordination with the NSW TB Control program. Prospective genotyping and genome sequencing support timely identification of clusters of infection and target public health follow-up of cases in order to identify potential transmission chains and minimise the risk of local tuberculosis transmission. All positive cultures are stored at the MRL for any additional microbiology tests.

Interferon Gamma Release Assays

Interferon Gamma Release assays (IGRAs) have limited role in the diagnosis of active tuberculosis and should not be a replacement for pathogen detection methods described above. However, there is a trend towards an increased use of IGRAs, especially in low-incidence countries, to supplement skin testing.

Peripheral venous blood is collected for IGRAs into special tubes provided by laboratories. The contents of the tubes should be thoroughly and vigorously mixed with the blood for at least five seconds immediately after collection. Incubation at 37°C ± 1°C should begin as soon as possible and within 16 hours of collection. Do not refrigerate or freeze the blood samples.

IGRAs are not serological tests and procedures for storage and transportation for blood samples for serology are not applicable for IGRAs. Important limitations of IGRAs should be noted:

- A negative result must be considered in conjunction with the patient's medical history, particularly for individuals with impaired immune function;
- Blood in special IGRA tubes should be promptly delivered to the testing laboratory and transported at room temperature.

Laboratory performance criteria

All positive culture and drug susceptibility test (DST) results affecting patient management should be phoned and faxed to the treating doctor and relevant TB Service (Chest Clinic) as soon as the results are available.

In addition, the NSW TB Control Program expects pathology providers to comply with the following criteria for mycobacteriology laboratory performance:

- Reporting of acid-fast microscopy results within 24-48 hours of specimen collection
- Genotypic drug resistance testing of sputum smear-positive cases within 72 hours of specimen collection
- Confirmation of *M. tuberculosis* complex isolates within 7 days of positive culture
- Reporting of phenotypic DST results (for at least isoniazid and rifampicin) within 21 days of positive culture.
- To perform MIRU-24 or WGS of all positive *M. tuberculosis* complex cultures within 3-6 months

All patients with sputum smear positive disease should receive a genotypic test to exclude likely drug resistance, within 3 days of initial sputum collection.

References

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2. NSW TB Program. *Sputum Induction Guidelines*. Sydney: NSW Health; 2015. Available from: www.health.nsw.gov.au/Infectious/tuberculosis/Pages/tb-sputum-induction-guidelines.aspx
3. Global Laboratory Initiative. *Mycobacteriology Laboratory Manual*. Stop TB Partnership; 2014. Available from: www.who.int/tb/laboratory/mycobacteriology-laboratory-manual.pdf

Further reading

National TB Advisory Committee. Guidelines for Australian Mycobacteriology laboratories. *Communicable Diseases Intelligence*. 2006; 30(1): 116-128. Available from: [www.health.gov.au/internet/main/publishing.nsf/Content/cda-cdi3001-pdf-cnt.htm/\\$FILE/cdi3001f.pdf](http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-cdi3001-pdf-cnt.htm/$FILE/cdi3001f.pdf)

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