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## **Revised Guidelines for Australian Laboratories performing Mycobacteriology Testing**

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Ivan Bastian, Lisa Shephard, Richard Lumb, and the National Tuberculosis  
Advisory Committee

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# Revised guidelines for Australian laboratories performing mycobacteriology testing

Ivan Bastian, Lisa Shephard, Richard Lumb, and the National Tuberculosis Advisory Committee

## Executive summary

Mycobacteriology laboratories play a key role in tuberculosis (TB) control by providing phenotypic and molecular diagnostics, by performing molecular typing to aid contact tracing, and by supporting research and similar laboratories in Australia's neighbouring countries where TB is prevalent. The National Tuberculosis Advisory Committee (NTAC) published a set of laboratory guidelines in 2006 aiming to document the infrastructure, equipment, staffing and work practices required for safe high-quality work in Australian mycobacteriology laboratories. These revised guidelines have the same aims and have been through a similar extensive consultative peer-review process involving the Mycobacterium Reference Laboratory (MRL) network, the Mycobacterium Special Interest Group (SIG) of the Australian Society for Microbiology (ASM), and other relevant national bodies.

This revised document contains several significant changes reflecting the publication of new biosafety guidelines and tuberculosis standards by various national and international organisations, technology developments – such as the MPT64-based immunochromatographic tests (ICTs) and the Xpert MTB/RIF assay – and updated work practices in mycobacteriology laboratories. The biosafety recommendations affirm the latest Australian/New Zealand Standard 2243.3: 2010 and promote a biorisk assessment approach that, in addition to the risk categorisation of the organism, also considers the characteristics of the procedure being performed. Using this biorisk assessment approach, limited manipulations, such as Ziehl-Neelsen (ZN) microscopy, MPT64 ICTs, and culture inactivation/DNA extraction for molecular testing, may be performed on a positive TB culture in a PC2 laboratory with additional features and work practices. Other significant changes include recommendations on the integration of MPT64 ICTs and novel molecular tests into TB laboratory workflows to provide rapid accurate results that improve the care of TB patients. This revised document supersedes the original 2006 publication. NTAC will periodically review these guidelines and provide updates as new laboratory technologies become available.

*Latest update: 29 April 2021. Refer to Appendix A for details.*

## Introduction

The incidence of tuberculosis (TB) in Australia is among the lowest in the world, with rates varying between 5.2 and 7.0 per 100,000 population since the mid-1980s.<sup>1</sup> However, TB remains a major global health problem. The World Health Organization (WHO) estimates that 10.4 million new cases of TB occurred in 2015 and 1.4 million people died from TB in that year.<sup>2</sup> Sixty-one per cent of these incident TB cases occurred in countries neighbouring Australia in the Western Pacific and South-East Asia regions.<sup>2</sup> Continued and improved TB control in Australia is therefore irrevocably linked to TB control in our region because nearly 90% of our cases occur in migrants.<sup>1</sup> Similarly, Australia's incidence of multidrug-resistant tuberculosis (MDRTB) is largely a reflection of the quality of TB treatment programs in our neighbouring countries. Of 22 MDRTB cases reported in Australia in 2013, twenty (91%) occurred in overseas-born individuals.<sup>1</sup>

Mycobacteriology laboratories play a key role in the control of TB and MDRTB in Australia. Laboratories provide:

- basic TB diagnostic services, such as microscopy, culture and direct detection by polymerase chain reaction (PCR);
- specialised TB diagnostic services, such as mycobacterial species identification, drug susceptibility testing, and rapid molecular detection of drug resistance;
- molecular epidemiological typing to support TB contact tracing;
- laboratory diagnosis of leprosy;
- specialised diagnostic services for the investigation of clinically-significant non-tuberculous mycobacteria (NTM) infections; and
- where possible and appropriate, undertake research into mycobacterial disease and/or support national TB laboratory services in

Australia's neighbouring countries where TB is prevalent.

The National Tuberculosis Advisory Committee (NTAC) published a set of guidelines for Australian mycobacteriology laboratories in 2006.<sup>3</sup> A revision of these guidelines is required to address several issues including: the publication of new biosafety guidelines and tuberculosis standards by various national and international organisations; technology developments such as the MPT64-based immunochromatographic tests (ICT) and the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA); and updated susceptibility testing breakpoints for *Mycobacterium tuberculosis* and non-tuberculous mycobacteria.<sup>4–24</sup>

## Formulation of the tuberculosis laboratory guidelines

The revised guidelines were drafted by NTAC and discussed at teleconferences and face-to-face meetings in 2016–2017. The five Mycobacterium Reference Laboratories in Australia were consulted as an 'expert user group' during this drafting process. Consultation was then undertaken with the ASM Mycobacterium SIG, the Public Health Laboratory Network (PHLN), the Royal College of Pathologists of Australasia (RCPA), Standards Australia, the Association of Biosafety for Australia & New Zealand (ABSANZ), and other interested parties.

This document supersedes the original 2006 publication. NTAC will periodically review these guidelines. Feedback on these guidelines is welcomed, as are suggestions for future changes, as the epidemiology of TB evolves and new laboratory technologies become available.

## Aims of the tuberculosis laboratory guidelines

These guidelines aim to document the infrastructure, equipment, staffing and work practice requirements for a modern mycobacteriology laboratory in Australia. The guidelines are based on published evidence (where available) and expert consensus. Areas of uncertainty are highlighted, particularly with regard to biosafety requirements for certain procedures, and a risk-based approach is proposed.

This NTAC publication is an advisory document recommending best practices for safe high-quality work in Australian mycobacteriology laboratories. These guidelines reaffirm and reiterate the biosafety requirements for Australian mycobacteriology laboratories as outlined in the latest Australian/New Zealand Standard 2243.3: 2010 *Safety in laboratories – Microbiological aspects and containment facilities*.<sup>24</sup> Laboratories must also comply with the National Pathology Accreditation Advisory Council (NPAAC) Standards for Pathology Laboratories, National Association of Testing Authorities (NATA), the *in vitro* diagnostic medical devices (IVDs) legislation implemented by the Therapeutic Goods Administration (TGA), and other relevant regulatory requirements.<sup>21–23</sup>

## Audience for the tuberculosis laboratory guidelines

The target audience for these revised guidelines remains unchanged from the 2006 version. The audience includes mycobacteriology staff, laboratory administrators, laboratory assessors, government authorities and the general public.

These guidelines are based on published evidence (where available) and expert consensus, and have been peer reviewed. Mycobacteriology laboratory staff can therefore use these guidelines as a benchmark tool for assessing their own laboratory performance.

Laboratory administrators must recognise the increasing investment required to provide a

modern high-quality mycobacteriology service meeting work health safety requirements. These guidelines attempt to provide some guidance on the minimum workload, staffing, equipment and infrastructure required to provide an acceptable service. Laboratory administrators can then decide whether their workload justifies the investment of providing these services.

These guidelines aim to provide laboratory assessors with a tool for assessing a mycobacteriology laboratory. However, while the safety requirements are obviously mandatory, it must be emphasised that reviewers should not consider any other single element as mandatory. Rather, a laboratory should be assessed across the spectrum of infrastructure, equipment, staffing, work practices and workload requirements, and must not be failed on any one deficiency.

Finally, this document aims to inform government authorities of the requirements for effective TB laboratory services so that adequate funds are available to meet these needs.

The Australian public can also be assured that high-quality mycobacteriology services continue to be provided throughout Australia.

## Related national and international guidelines

The original 2006 guidelines cited the Australian/New Zealand Standard 2243.3 *Safety in laboratories – Microbiological aspects and containment facilities*; the NPAAC Standards for Pathology Laboratories; two US guidelines; and the New Zealand compendium entitled “Guidelines for tuberculosis control in New Zealand”, which included a chapter for the mycobacteriology laboratory. In the last decade, at least fourteen relevant international documents have been published.<sup>4–17</sup>

## Significant developments since the 2006 guidelines

### Risk-based approach to biosafety

The 2006 NTAC guidelines proposed that TB laboratories undertaking more than 5,000 cultures per year, performing susceptibility tests, or knowingly handling MDRTB strains, should have PC3 facilities.<sup>3</sup> The Australian/New Zealand Standard 2243.3: 2010 subsequently adopted those recommendations.<sup>24</sup>

There has been an evolution in biosafety guideline development over the last decade to a risk-based approach. Previously, an organism was assigned a risk group based on its virulence, transmissibility, and the availability of treatments or vaccinations. A specific laboratory physical containment (PC) level (with a stipulated suite of infrastructure, equipment and work practice requirements) was then designated for the organism in a formulaic match that took no account of the actual procedure being performed with the organism or the inherent risks of that procedure. A shift from this formulaic approach occurred in 2008 when the European Committee for Standardization published the standard CWA 15793, which promoted a biorisk management system.<sup>25</sup> The WHO TB Laboratory Safety Manual (2012) and the 2009 publication 'Biosafety in Microbiological and Biomedical Laboratories' from the US Centers for Disease Control (CDC) have subsequently incorporated risk assessment approaches that also consider the characteristics of the procedure being performed, such as: the organism concentration, suspension volume, procedural complexity, and risk of aerosolisation.<sup>6,8</sup>

A retrospective study from South Korea by Kim *et al.* provides one of the few estimates of the risk of various TB laboratory procedures.<sup>26</sup> They compared the incidence of TB among laboratory workers performing microscopy, culture or drug susceptibility testing (DST) with the incidence among managerial/clerical workers. Compared to non-laboratory staff, the relative risk of TB among microscopy, culture or DST workers was

1.4 (95% CI, 0.2–10.0), 2.0 (0.2–13.3) and 21.5 (4.5–102.5), respectively.<sup>26</sup> Scientists performing DST, which is a complex task involving high concentrations of potentially drug-resistant *M. tuberculosis*, require appropriate infection controls to undertake their work safely.

In retrospect, the 2006 NTAC laboratory guidelines represented a risk-based approach proposing PC3 facilities for TB laboratories undertaking more than 5,000 cultures per year, performing susceptibility tests, or knowingly handling MDRTB strains. These triggers for requiring PC3 facilities remain unchanged in this revised document. Institutions are encouraged to implement biorisk management and to conduct risk assessments on their laboratory procedures as outlined in the standard CWA 15793.<sup>25</sup>

There have been new technologies developed over the last decade such as MPT64-based ICTs and the Xpert MTB/RIF assay (described below) that can be performed on positive automated broth-based cultures such as Mycobacterium Growth Indicator Tubes (MGIT; Becton Dickinson). This revised guideline recommends that laboratory managers conduct a risk assessment within a "CWA 15793-like" framework about performing certain limited manipulations on positive TB cultures in a PC2+ facility – ie. a PC2 laboratory with a biosafety cabinet (BSC), directional airflows, and an 'anteroom' (so that the TB laboratory cannot be directly accessed from a public corridor). These limited manipulations include: Ziehl-Neelsen (ZN) microscopy, MPT64 ICTs, and culture inactivation/DNA extraction for a molecular test such as an Xpert MTB/RIF or a line-probe assay (LPA). Similarly, a laboratory manager may conduct a risk assessment about performing primary processing and initial culture of specimens from a known MDRTB patient. Positive cultures from such patients must have no more than a ZN stain performed before immediate referral to a PC3 reference laboratory.

Aerosol-generating activities represent the most significant risk for laboratory-acquired infections. The WHO TB Laboratory Safety Manual

provides specific recommendations for minimising aerosol production (page 21, reference 8). These recommendations include leaving tubes to sit undisturbed for  $\geq 10$  minutes to allow aerosols to settle after vortexing or shaking.<sup>8</sup> This interval should be extended to 15 minutes when handling high concentrations of TB bacilli (eg. positive MGIT tubes, DST manipulations).<sup>8</sup>

### MPT64 immunochromatographic tests

Three commercial ICTs are available that detect MPT64, a secretory protein specific to the *M. tuberculosis* complex, in liquid and solid cultures.<sup>18,27</sup> These assays are simple to perform, have a turnaround time (TAT) of 30 minutes, and allow rapid identification of *M. tuberculosis* in positive liquid cultures such as MGIT. A meta-analysis of commercial ICTs reported a pooled sensitivity of 97% (95% CI, 96–97%) and a pooled specificity of 98% (95% CI, 98–99%).<sup>27</sup> False-negative results occur for some, but not all *Bacillus Calmette- Guérin* (BCG) strains, some *M. bovis* isolates, and with *M. tuberculosis* isolates containing MPT64 gene mutations.<sup>18,28</sup> Rare false-positive results have been reported with NTM, including *M. kansasii*, *M. gastri* and *M. terrae*.<sup>18,28</sup> This revised guideline recommends that PC3 laboratories (and suitably risk-assessed PC2+ laboratories) should perform MPT64 ICTs (or a similar rapid identification test such as a molecular assay or MALDI-TOF mass spectrometry) within 24 hours of the first positive mycobacterial culture from a patient suspected of TB. However, the laboratory's testing algorithm must contain a secondary (molecular) identification test to detect MPT64-negative *M. tuberculosis* isolates.

### Xpert MTB/RIF and other molecular assays

The Xpert MTB/RIF assay uses real-time (rt) PCR technology to detect TB and rifampicin resistance concurrently.<sup>29</sup> Extraction, amplification and detection processes occur in an automated closed cartridge system. Results are available within 2 hours. Table 1 lists the performance characteristics of the assay on different

specimen types. This assay has revolutionised TB diagnostics in high-incidence low-resource settings with >16 million tests performed in 122 countries since 2011.<sup>30</sup> In the US, the Xpert MTB/RIF assay is approved for use on an unprocessed sputum specimen or concentrated respiratory sediment. Based on a series of systematic reviews and expert opinion, WHO has expanded these specimen types to include CSF (strong recommendation, very low-quality evidence), lymph node and other tissues (conditional recommendation, very low-quality evidence).<sup>31</sup> The WHO expert review noted 'substantial heterogeneity' in the performance characteristics of the Xpert MTB/RIF assay for detecting extrapulmonary TB depending on the specimen type. For example, lymph node tissue/aspirate and cerebrospinal fluid (CSF) provided reasonable performance (ie. pooled sensitivity compared with culture of 84.9% and 79.5%, respectively) whereas pleural fluid was deemed a 'suboptimal specimen' (ie. pooled sensitivity compared with culture of 43.7%).<sup>31</sup>

The US Food and Drug Administration has recently approved an additional indication for the Xpert MTB/RIF assay, which is to test 1–2 respiratory specimens to inform decision-making about lifting airborne precautions (AP) for patients with suspected pulmonary tuberculosis.<sup>32</sup> Historically, the US CDC has required three serial acid-fast bacilli (AFB) negative smears collected 8–24 hours apart to discontinue AP precautions. Similar protocols are used in Australian hospitals. However, three studies have shown that negative Xpert MTB/RIF assay results from 1–2 sputum specimens are comparable with the results of two or three negative acid-fast sputum smears for this purpose (summarised in reference 32). The need for 1–2 Xpert MTB/RIF tests depends on individual clinical circumstances and institution guidelines.

Recently an improved Xpert assay, the Xpert MTB/RIF Ultra has been released to market and is expected to replace the conventional Xpert assay in the lifetime of this guideline. The Ultra assay is more sensitive than the conventional assay which leads to improved diagnostic yield

**Table 1: Sensitivity and specificity of nucleic acid tests in clinical specimens**

	Smear-positive respiratory		Smear-negative respiratory		Extrapulmonary	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Xpert MTB/RIF <sup>29</sup>	98–100%	>98%	57–83%	99%	53–95%	98–99.6%
Line Probe Assay <sup>33,34</sup>	93.4%	85.6%	N/A	N/A	N/A	N/A
In-house NAAT <sup>35</sup>	Sensitivity: 84–100% Specificity: 83–100%					

N/A, not applicable; NAAT, nucleic acid amplification test

in smear negative TB cases, children, human immunodeficiency virus (HIV) infected subjects and especially for the diagnosis of TB meningitis (TBM). The assay is endorsed by WHO as an alternative for the conventional Xpert assay for all indications.<sup>74</sup>

In 2008, LPA became the first molecular method endorsed by WHO for detection of *M. tuberculosis* and drug resistance from smear-positive patients at risk of MDRTB.<sup>33</sup> Line probe assays are based on the reverse hybridisation principle. Specific oligonucleotides are immobilised at known locations on a membrane strip and are hybridised under strictly controlled conditions with the biotin-labelled PCR product. Commercially-available LPAs include the INNO-LiPA Mycobacteria (Fujirebio Inc, Ghent, Belgium) and the GenoType MTBC (Hain Lifesciences, Germany) for mycobacterial species identification and differentiation within the *M. tuberculosis* complex, respectively. The MDRTB<sup>plus</sup> assay (Hain Lifesciences) allows direct detection of *M. tuberculosis*, isoniazid and rifampicin resistance from smear-positive pulmonary specimens.<sup>34–36</sup>

In addition to commercial assays such as Xpert and LPA, some laboratories have developed and validated in-house nucleic acid amplification tests (NAATs) for detection of *M. tuberculosis* and associated rifampicin resistance. A subsequent section entitled ‘Guidelines for Nucleic Acid Amplification Tests’ will describe the recommended application of these TB molecular tests.

Despite the above developments in molecular technologies, phenotypic culture and sus-

ceptibility testing currently remain the ‘gold standard’ TB diagnostic tests with NAATs being supplementary tests. Two impending developments are likely to reverse this paradigm before the next formal revision of these NTAC laboratory guidelines.<sup>36</sup> Firstly, Cepheid and Abbott Molecular have both announced next-generation NAATs with reported levels-of-detection approaching that of culture. Secondly, whole genome sequencing (WGS) could replace phenotypic DST but method standardisation, database development and elucidation of all resistance gene determinants is required. NTAC will monitor these developments in molecular technology and will develop suitable updated recommendations when appropriate.

### Pyrazinamide and other susceptibility testing

The 2006 NTAC guidelines recommended against routine DST for pyrazinamide (PZA) based on the low prevalence of PZA resistance in Australia and the problematic nature of PZA DST.<sup>3</sup> Pyrazinamide is an increasingly important drug, as part of the recommended short-course treatment regimen for drug-susceptible TB, but is also included in established and novel MDRTB treatment regimens.<sup>37</sup> Multiple international authorities now recommend PZA DST as part of routine first-line testing.<sup>7,9–11</sup> Unfortunately, PZA DST still remains problematic for two reasons. First, the requirement for testing at low pH is itself inhibitory to most *M. tuberculosis* isolates. Second, the MGIT 960 method is prone to false-resistant results due to high inocula.<sup>38</sup> Molecular detection of PZA resistance is confounded by the heterogenous mutations encoding resistance

along the 558 bp length of the *pncA* gene, the gene encoding pyrazinamidase, and the presence of other PZA-resistance mechanisms.<sup>39,40</sup>

While recognising the above limitations, these revised NTAC guidelines encourage laboratories to perform PZA DST routinely as a first-line test. Phenotypic DST by the MGIT PZA test may be used as a 'screening' test for PZA susceptibility.<sup>40</sup> An initial PZA-resistant result could then be followed by a repeat MGIT test and *pncA* gene sequencing to confirm the presence of PZA resistance.<sup>39,40</sup> Increased experience with PZA DST and future innovations will hopefully lead to improvements in PZA testing.<sup>41</sup> In the meantime, laboratories and clinicians must recognise the vagaries of PZA DST, and that WHO considers the use of PZA (as an ancillary drug) is an acceptable practice even when a laboratory result demonstrates resistance.<sup>42</sup>

Other DST changes from the 2006 NTAC guidelines include updated breakpoints for *M. tuberculosis* susceptibility testing and adoption of the broth microdilution methodology for rapid-growing mycobacteria (RGM) as described by the Clinical and Laboratory Standards Institute (CLSI).<sup>16</sup> Most importantly, a checklist is provided for laboratories to exclude contamination as the cause of false-resistant results, particularly when reporting a putative MDRTB or extensively drug-resistant (XDR) TB case.

### Recent changes to international guidelines for smear microscopy

A revised and updated handbook for the laboratory diagnosis of tuberculosis by smear microscopy was released by the Global Laboratory Initiative (GLI) in 2013.<sup>43</sup> Several changes have been made to the recommended formulations of the reagents to optimise the reliability of TB stains. For ZN microscopy, the changes are:

- Carbol fuchsin (CF) concentration increased to 1% (previously 0.3%);
- CF staining time increased to 10 minutes;

- 6% aqueous hydrochloric acid as an alternative decolouriser; and
- 0.1% methylene blue as the counterstain (previously 0.3%).<sup>43</sup>

For fluorescence microscopy, the changes are:

- Auramine concentration of 0.1%;
- Staining time increased to 20 minutes;
- 0.3% methylene blue as an alternative counterstain to potassium permanganate (KMnO<sub>4</sub>);
- Quantitation scale recalibrated to correct an historical error; and
- Confirmation of 'scanty' AFB positive smears by ZN is not required.<sup>43</sup>

These guidelines support the methods recommended in the updated WHO microscopy handbook.<sup>43</sup> However, mycobacteriology laboratories may continue employing their established methods provided high-quality CF is used and satisfactory performance is documented in an external quality assurance program (QAP).

## Guidelines for a laboratory performing smear microscopy

### Biosafety considerations

The most common specimen for TB microscopy is sputum, which is usually viscous in character. There is a low risk of generating infectious aerosols, and the bacterial load of AFB is low. Similarly, tissues, biopsies and specimens from usually sterile sites, if TB-culture positive, are usually smear negative and carry a low infectious risk. The retrospective study by Kim *et al* described previously found that microscopy technicians were not at significantly increased risk of TB compared to non-laboratory workers.<sup>26</sup> WHO and the International Union Against TB and Lung Disease (Union) therefore consider smear preparation a low-risk procedure.<sup>8</sup> Nonetheless, all suitable safety measures must be provided in a high-income country such as Australia with a low incidence of TB.

### General laboratory facilities, equipment and work practices

Laboratories performing smear microscopy must comply with the requirements of a PC2 facility as described in the Australian Standard.<sup>24</sup> The following (additional) requirements are emphasised:

1. The smear preparation procedure must be performed in a Class I or Class II BSC.
2. The operator must wear gloves and a long-sleeved gown or coat where the glove and sleeve cuff overlap.
3. Any manipulation involving vortexing, shaking, mixing or sonication must be performed in the BSC and a period of at least 10 minutes elapse before the container is opened within the BSC.
4. If a concentrated smear is being prepared, a centrifuge with sealed rotors or safety cups must be used and must be capable of reaching and maintaining 3,000 ×g to sediment

the AFB reliably.<sup>44</sup> The specimen must not be heated above 37 °C during centrifugation.

5. Access to the laboratory must be limited to personnel and persons specified by the laboratory management.
6. Packaging of specimens for shipment by a public carrier to the culture laboratory must comply with International Air Transport Association regulations (summarised in AS/NZS 2243.3) and the relevant Australian Standard.<sup>24,45</sup> Individual courier companies may have additional requirements that must be met otherwise the shipment may be delayed or refused.

## Requirements specific to a laboratory performing TB smear microscopy

The following work practices are recommended for laboratories performing TB smear microscopy:

1. Smear results should be available within 24 hours of specimen reception. Results should be available within 24 hours even on weekends for specimens considered urgent; results for non-urgent routine requests should be available on the following Monday. The treating doctor and the laboratory director/clinical microbiologist should liaise to decide whether weekend specimens are urgent or non-urgent. Urgent smear microscopy after hours may, by necessity, need to be performed directly on unprocessed sputum, with sputum concentration and decontamination for culture occurring later during routine hours.
2. Specimens for cultures should be transported to the relevant laboratory within the next working day.
3. An Xpert MTB/RIF assay (or similar NAAT) must be performed on a smear-positive specimen from a TB suspect within 72 hours, either in the testing laboratory or by referral to a larger central laboratory.
4. A positive- and a negative-control smear should be included with each batch of smears.
5. Positive results should be quantified using the Union/WHO scale.<sup>43</sup>
6. A laboratory performing TB smear microscopy should process a minimum of 10 requests per week to maintain expertise. A technician should process and read no more than 25 ZN smears per day on average. Up to 75 slides per day can be read if a fluorochrome stain is used.
7. The staining reagents must be labelled with their identity, concentration, preparation date, expiration date, initials of the technician who prepared the reagent, and any relevant safety symbols. Laboratories are reminded of the recent changes recommended by GLI to the reagent formulations for ZN and fluorescent microscopy.<sup>43</sup> However, mycobacteriology laboratories may continue employing their established methods provided high-quality CF is used and satisfactory performance is documented in an external QAP.
8. The staining method should be clearly described in the laboratory method manual, which should also list the remedial actions if the positive or negative control slide fails.
9. Larger laboratories that process many specimens (and perform cultures) may use a fluorochrome stain. It is not necessary for smears from new smear-positive patients to be checked by ZN stain.
10. The laboratory must participate in an external QAP. The RCPA program sends 8–10 AFB smears per year. Quantitation errors are of minor significance.<sup>46</sup> Low false-negative results are understandable if the QAP sends a slide with 1–9 AFB/100 fields. Any low or high false-positive result or any high false-negative result should trigger immediate remedial action.<sup>46</sup>

## Requirements for a sputum collection area

Some laboratories may be responsible for collecting TB sputum specimens. The laboratory must therefore ensure that a high-quality specimen is collected, suitably labelled, and that the collection is performed safely.

Whereas smear preparation is a low-risk procedure, sputum collection from a smear-positive patient is a high-risk procedure and must be performed in the correct setting.<sup>8</sup> NTAC and the US Centers for Disease Control and Prevention have updated extensive guidelines on reducing TB transmission in the health-care setting.<sup>47,48</sup>

The NTAC document has a section devoted to 'specimen collection' and the reader is referred to that publication.<sup>47</sup> Points of particular relevance to the laboratory are:

- the clinical service and/or laboratory should provide an instruction form to the patient describing the method of producing a good sputum specimen, the timing of the collection, and the handling of the specimen (eg. refrigeration at 4 °C); and
- appropriate containers (sterile and with a multi-thread leak-proof cap) should be provided to the patient.

**Table 2: Quantitation scale for brightfield microscopy (Ziehl-Neelsen)<sup>43</sup>**

Smear microscopy result	Minimum number of high power fields to be read	Interpretation
Negative	100	No acid-fast bacilli detected
1–9 AFB	100	Record exact number of bacilli
10–99 AFB	100	1+
1–10 AFB/field	50	2+
>10 AFB/field	20	3+

**Table 3: Quantitation scale for fluorescence microscopy (Auramine)<sup>43</sup>**

Smear microscopy result (200x total magnification)	Smear microscopy result (400x total magnification)	Interpretation
No AFB in one length <sup>a</sup>	No AFB in one length	No acid-fast bacilli detected
1–4 AFB in one length	1–2 AFB in one length	Confirmation required <sup>b</sup>
5–49 AFB in one length	3–24 AFB in one length	Scanty
3–24 AFB in one field	1–6 AFB in one field	1+
25–250 AFB in one field	7–60 AFB in one field	2+
>250 AFB in one field	>60 AFB in one field	3+

a One length is equivalent to 2 cm.

b Confirmation required by another technician or prepare another smear, stain, and read.

## Guidelines for Laboratories performing Mycobacterial Cultures

### Biosafety considerations

For mycobacterial culture, the processing and concentration of specimens for inoculation on to primary media or for sample preparation for LPA is considered to have a moderate risk. Although there is usually a low concentration of infectious particles, specimens are liquefied during the processing procedure, increasing the chance of generating infectious aerosols. In the retrospective study by Kim *et al*, laboratory staff performing TB culture procedures ‘only’ had a relative risk of 2.0 (95% CI 0.2–13.3) compared with non-laboratory staff.<sup>26</sup>

A PC2 laboratory with additional equipment and work practices would appear to be an appropriate facility for performing the large majority of TB cultures in Australia. Laboratories undertaking more than 5,000 cultures per year, performing susceptibility tests, or knowingly handling MDRTB strains should be undertaking TB culture within a PC3 facility.

### General laboratory facilities, equipment and work practices

1. The TB culture laboratory must be in a self-contained room physically separated from other areas. The laboratory should be divided into areas where ‘clean’ activities (administration, microscopy, staining, storage of consumables and reagents) and ‘dirty’ activities (processing of specimens, handling cultures, BSC, centrifuge, incubators) are located.
2. The ‘clean’ area should be near to the entry/exit point of the laboratory and have a hand-washing station and gowns hooks. The ‘dirty’ area must be located away from the entry/exit point.
3. Access to the TB laboratory must be limited to staff trained to work in the area. Access should be restricted by lockable doors.

4. A pressure steam steriliser must be available for decontaminating laboratory waste, preferably within the TB laboratory but otherwise within the laboratory facility. The WHO TB laboratory biosafety manual defines ‘waste’ as any item that is being discarded.<sup>8</sup> Non-sterilised waste taken out of the TB culture laboratory must be double-bagged, placed within a container with a lockable lid, and taken directly to the pressure steam steriliser.
5. A directional air flow from the entry/exit point to the ‘dirty’ area shall be maintained by extracting room air. Recirculation is permitted but not into areas outside the PC2–PC3 facility.
6. All procedures must be performed in a Class I or Class II biosafety cabinet (BSC). The BSC must undergo at least an annual maintenance check and be certified for use.
7. For personal protective equipment, staff must wear gloves and a long-sleeved gown where the glove and sleeve cuff overlap. These personal protection items must not be worn outside of the TB laboratory.
8. Laboratory coats must not be used for TB culture as they do not provide adequate coverage to the user.
9. N95 (P2) respirators should be included in the spill kit and worn should a spill event occur outside of the BSC-II. N95 respirators should be available to use when performing high-risk activities. Such respirators are not a substitute for a poorly functioning BSC. Local risk assessments are recommended to determine the appropriate level of respiratory protection required for each activity. Respirators must be correctly fitted, which may be achieved by fit testing.<sup>47</sup> Wearers must be trained in respirator selection, performing a ‘fit check’, donning and removal, proper use, and limitations.
10. Any manipulation of specimens involving vortexing, shaking, mixing or sonication

must be performed in the BSC and a period of at least 10 minutes must elapse before the container is opened in the BSC.

11. When cultures are vortexed, shaken, or sonicated, at least 15 minutes should elapse before opening the container.<sup>8</sup>
12. A centrifuge with sealed rotors or safety cups must be used and must be capable of reaching and maintaining 3,000 ×g to reliably sediment AFB.<sup>44</sup> The specimen must not be heated above 37 °C during centrifugation.

### Requirements specific to a laboratory performing TB culture

1. A scientist with a university degree (or equivalent training) should be responsible for the TB laboratory. All staff working in the TB laboratory should have been suitably trained and have evidence of ongoing training. A clinical microbiologist should have active input into the laboratory planning, procedures, and supervision, and should be available to communicate any positive culture results, where necessary.
2. To maintain technical competency, a TB culture laboratory should process at least 20 specimens for culture per week.
3. Ideally, specimens should be processed on each day of the working week. Smaller laboratories culturing 20–50 specimens per week may choose to process cultures 3–4 times per week. In these circumstances, smears are to be prepared and read daily.
4. All specimens should be inoculated in a liquid culture system with or without an additional inoculation on solid media. Liquid culture provides appreciably faster TATs than those achieved by culture on solid media. Liquid culture systems should therefore be used by default. Various authorities recommend that each specimen should also be inoculated onto solid media to detect strains that may not grow in broth.<sup>9–12</sup> Growth on solid media only in comparative studies may be due to the ‘splitting’

of samples with low AFB counts across multiple media and may not be a major problem if all of the sediment is inoculated into the broth. The US Association of Public Health Laboratories (APHL) recommend further study of the cost-effectiveness of routine inoculation of solid media and that individual laboratories develop their own policy decisions based on their local data.<sup>5</sup> Selective use of solid media may therefore be acceptable based on local data assessments (eg. on all sterile site specimens such as tissues or CSF, and on any smear-positive specimen).

5. Processing of multiple specimens from each presumptive TB case increases the sensitivity of culture.
6. Specimens from skin, lymph nodes and abscesses that may contain pathogenic nontuberculous mycobacteria (NTM) should also be inoculated onto/into additional media for incubation at 30 °C.
7. Specimens from sterile sites (eg. CSF, biopsies) usually do not require decontamination and should be inoculated directly into liquid and solid-media.
8. Environmental samples may also be tested for the presence of NTM, most commonly endoscopic instrument washings. In addition, PHLN have published recommendations for mycobacterial culture of water from heater cooler units (HCUs) used for cardiopulmonary bypass and other applications.<sup>49</sup>
9. The inclusion of positive- and negative-culture controls with every batch of specimens for culture is not necessary. Positive controls represent a potential source of contamination and should only be included when a new batch of media is used. Negative controls will only reliably detect gross cross-contamination that will be self-evident. Low-level contamination will be inconsistent and may not be detected in negative-control vials. Recording of background bacterial contamination rates is far more important.

10. Contamination rates should be recorded. For liquid culture systems, contamination rates of 8–10% are acceptable, and represent the best balance between excessive contamination and overly stringent decontamination (which risks false-negative culture results). For solid media, contamination rates of 3–5% are acceptable.

11. Laboratories must be alert to cross-contamination between specimens resulting in false-positive results. In US and European studies, 1–4% of cultures may be false-positive cultures and the consequences for the patient may be substantial.<sup>50</sup> Laboratory cross-contamination should be considered in the following circumstances:<sup>51</sup>

- a single smear-negative *M. tuberculosis*-culture-positive specimen when other samples from the patient are smear- and culture-negative;
- the patient's clinical presentation or course is inconsistent with TB;
- unusual clustering of positive-culture results processed on the same day;
- isolates with unusual DST profiles that were processed on the same day;
- ≤ 5 colonies grow on solid media, or time to growth detection is > 30 days in automated broth cultures, or discordant results are obtained when solid- and broth-based media are inoculated with the same specimen.

Suspicious of laboratory cross-contamination events should be investigated by:

- reviewing the laboratory workbook for other culture-positive specimens processed at the same time;
- reviewing the patient's history, radiological investigations, clinical course, and response to therapy;
- genotyping of the suspicious isolates which may demonstrate identical profiles to laboratory

control strains (eg. H37Rv) or to isolates from epidemiologically-unrelated patients processed on the same day; and

- reviewing the laboratory procedures.

12. Automated liquid-based cultures are incubated and monitored continuously for 6 weeks. Non-automated liquid-based cultures should be read every 2–3 days for weeks 1–3, and weekly thereafter for at least 6 weeks but longer if required, depending on the specimen type and smear result. Solid media should be read twice weekly for weeks 1–4 then weekly thereafter for at least 8 weeks, but longer if required.

13. All positive broth-based cultures must be: ZN stained, sub-cultured to solid media (to detect mixed mycobacterial growths), and sub-cultured to blood agar (to detect bacterial contamination). Repeat positive cultures must have a ZN stained smear performed to confirm presence of AFB, and sub-cultured where appropriate.

14. TB laboratories working in PC3 facilities (or suitably-risk-assessed PC2+ facilities) should perform MPT64 ICTs (or a similar rapid identification test such as a molecular assay) within 24 hours of the first positive mycobacterial culture from a patient. However, the laboratory's testing algorithm must contain a secondary (molecular) identification test to detect MPT64-negative *M. tuberculosis* isolates.

15. *M. tuberculosis* and NTM may also be identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.<sup>52,53</sup> This technology has demonstrated rapid and accurate identification but users are required to optimise protein extraction techniques (particularly from liquid cultures) and to develop a customised spectral library. Most importantly, MALDI users must confirm that their extraction technique inactivates pathogenic *M. tuberculosis* so that target plates can be safely moved from the (PC3) mycobacteriology laboratory to the mass spectrometer.

16. Laboratories performing cultures but referring isolates for identification and DST must send all positive cultures to the reference laboratory within 48 hours of culture positivity. The sample should be sub-cultured to a blood agar plate and incubated for a minimum of 24 hours to exclude bacterial contamination before sending to the higher-level laboratory. Shipment of isolates must comply with relevant national and state regulations.<sup>24,45</sup> Depending on the transport regulations, the isolate may be sent in liquid or on solid media. The isolate must be accompanied by the original request form and documentation of all relevant clinical and laboratory information (eg. patient details, original specimen type, AFB smear result, associated histological investigations that may have been performed on the same specimen).
17. Laboratories should aim to report positive MTBC cultures within an average of 14–21 days from time of specimen reception.<sup>9</sup> These TATs are achievable using broth-based culture systems.
18. All positive culture and DST results that will affect patient management should be phoned and sent electronically and/or in printed form to the treating doctor and the responsible state or territory TB control unit as soon as the results become available. For example, the initial results on all new patients, relapses and failure cases must be phoned and sent directly to the treating doctor. Repeat results on subsequent specimens from the same episode can be sent in printed form.
19. *M. tuberculosis* is a notifiable disease in all states and territories (as are NTM infections in some states). Microbiological laboratories performing TB cultures should ensure that they, or the reference laboratory to which their cultures are referred, comply with the local jurisdictional requirements for laboratory notifications.
20. All primary MTBC isolates should be retained for at least six months by the referring laboratory and for at least five years by the reference laboratory.
21. Reference laboratories should also provide, directly or through collaborative agreements, access to molecular epidemiological tools so that outbreak strains and laboratory cross-contamination episodes can be recognised promptly. Mycobacterial interspersed repetitive-unit-variable-number tandem repeat (MIRU-VNTR) genotyping is the current molecular technique employed widely in Australia and New Zealand. However, MIRU-VNTR will be supplanted by whole genome sequencing.<sup>36</sup>
22. Laboratories performing TB cultures must participate in a recognised QAP program. The RCPA QAP program distributes eight specimens for mycobacterial culture per year. Results should be reviewed by the institution's Quality Services section, and laboratory procedures reviewed when any false-positive or false-negative culture results occur.
23. Laboratories performing TB cultures should liaise closely with their state Mycobacterium Reference Laboratory (MRL). This liaison may be demonstrated by consultation over positive cultures and/or by attendance at clinical meetings.
24. A laboratory performing mycobacterial cultures but not meeting the minimum recommended workload (ie. 20 specimens for culture per week), not fulfilling QAP or other requirements must consider referring their mycobacteriology workload to a larger central facility.
25. The ASM Special Interest Groups for Media Quality Control and Mycobacteriology have developed guidelines for assuring the quality of solid media.<sup>54</sup> It is recommended laboratories comply with this document.

## Requirements for non-tuberculous mycobacteria cultures

Non-tuberculous mycobacteria are ubiquitous in the environment, and are found in water, soil, dust, and air. There are well over 100 NTM with formally approved names, and an unknown number of informally-described NTM within the genus *Mycobacterium*.<sup>55</sup> Isolation of an NTM does not necessarily imply that the organism is of clinical significance, and deciding whether an NTM is relevant is a combination of clinical, radiological, and microbiological determinations.<sup>17,56</sup>

In Australia, the culture and identification of NTM represents an increasing proportion of the workload for the MRL network. Moreover, susceptibility testing protocols are available for only a small number of NTM (*Mycobacterium avium* complex, *M. kansasii*, *M. marinum*, and rapidly-growing mycobacteria including the *M. abscessus* complex, *M. chelonae* and *M. fortuitum*).<sup>16</sup> For the slowly-growing mycobacteria specified, DST protocols are limited to one or few anti-mycobacterial agents.

Identification (with or without susceptibility testing) should only be performed on clinically-relevant isolates and must not be performed on every NTM isolate as the majority represent either colonisation or contamination. Microbiological criteria associated with clinical relevance include:

- positive culture results from at least two separate expectorated sputum samples; or
- culture from at least one bronchial wash or lavage; or
- transbronchial or lung biopsy with NTM and histopathological features of mycobacterial infection/disease; or
- NTM isolated from a normally sterile site (such as blood, cerebrospinal fluid or tissues); or
- at least one NTM isolated from patients with serious immunosuppression.<sup>17</sup>

Interpretation of the significance of NTM cultures is an excellent example of the necessity for collaboration between clinicians and the laboratory. The culture results must be interpreted in combination with the patient's clinical presentation and radiological features. Clinicians should also liaise with the laboratory when an unusual mycobacterium is identified because some NTM (eg. *M. kansasii*, *M. szulgai*) are recognised as often pathogenic whereas other NTM (eg. *M. gordonae*) are rarely pathogenic.<sup>56</sup>

Identification of NTMs may be achieved by a combination of molecular methods, such as PCR restriction analysis, DNA probes (eg. AccuProbe system, Hologic Gen-Probe, San Diego, CA), specific gene sequencing (eg. targeting 16S rRNA, *hsp65*, *rpoB*, ITS, *sodA*) or WGS.<sup>55</sup> High-performance liquid chromatography (HPLC) of cell wall mycolic acids still has proponents, particularly in the US, but increasing recognition of new mycobacterial species sharing common HPLC profiles has reduced its discriminatory power and has 'dated' this technology.<sup>55</sup> As described above, MALDI-TOF may become another established mycobacterial identification methodology when optimised.<sup>52,53</sup>

NTM isolates should be identified to species level except for *M. intracellulare*, *M. avium* and *M. chimaera* and related organisms where identification to the *M. avium* complex level (MAC) is considered sufficient (unless the investigation is for HCU-related *M. chimaera* infections). *Mycobacterium abscessus* should be identified to subspecies level (ie. *M. abscessus* subspecies *abscessus*, *bolletii* or *massiliense*). Specific identification helps predict treatment response for this complex. *Mycobacterium abscessus* subspecies *abscessus* has a full-length functional *erm41* gene which is associated with inducible clarithromycin resistance. In contrast, *M. abscessus* subspecies *massiliense* has a partial *erm41* gene deletion preventing inducible resistance and thus has better outcomes with macrolide based treatment.<sup>57</sup> Unfortunately, identification of subspecies within the *M. abscessus* complex may be problematic for two reasons. First, taxonomists have argued whether there are two or three subspecies within this complex.

Second, WGS studies demonstrate horizontal gene transfer within the complex producing chimera between the subspecies<sup>58,59</sup> Sequencing of one gene locus is therefore inadequate for accurate subspecies identification within the *M. abscessus* complex.<sup>59,60</sup> Sequencing of two loci (eg. *rpoB* and *hsp65*) is the current standard.<sup>60</sup> The future gold-standard may be WGS or sequencing a selection of the 66 genes unique to each subspecies.<sup>58</sup>

Recommendations on the investigation of leprosy (*M. leprae*) are beyond the scope of this document. The Public Health Laboratory Network (PHLN) have published a laboratory-case definition that provides suitable advice.<sup>i</sup>

### **Non-tuberculous mycobacteria in individuals with cystic fibrosis**

The US Cystic Fibrosis (CF) Foundation and the European Cystic Fibrosis Society have developed recommendations for the screening, investigation, diagnosis and management of NTM pulmonary disease in individuals with CF.<sup>57</sup> In this population, NTM has emerged as a significant threat to the health of an individual, and diagnosis and treatment remains a challenge.

In individuals with CF, cultures for NTM should be performed annually in spontaneously expectorating individuals. Screening is not required in those who are unable to produce a sputum in the absence of clinical features suggestive of NTM pulmonary disease. When NTM is suspected in individuals with CF, smears and cultures from sputum, induced sputum, bronchial washing or bronchoalveolar lavage samples may be used to confirm NTM disease.<sup>57</sup>

Inoculation of solid and liquid media is recommended for respiratory tract samples from CF patients. Samples should be processed within 24 hours of collection to optimise decontamination and detection of NTM. Cultures should be incubated for a minimum of 6 weeks.<sup>57</sup>

The identification and susceptibility testing recommendations for NTM described elsewhere in this document also pertain to the handling of such isolates from CF patients.

A five-year NHMRC project is underway to investigate the incidence, characteristics and management of NTM infections in a cohort of 1,800 CF patients across Australia. Several laboratory-based ancillary studies will investigate various strategies for optimising NTM investigations in this patient population, such as optimised re-decontamination protocols and the use of novel solid media for isolation of NTM from direct sputum specimens.<sup>61</sup> The results of such studies will inform updates to these NTM laboratory guidelines.

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i Available at: <http://www.health.gov.au/internet/main/publishing.nsf/content/cda-phlnccd-leprosy.htm>

## Guidelines for laboratories performing susceptibility tests

### Biosafety considerations

Drug susceptibility testing is considered the highest risk activity in the TB laboratory because aerosols are easily generated during complex manipulations with high-titre liquid cultures. Unsurprisingly, the retrospective study by Kim *et al* found that DST workers had a significantly-higher risk of active TB disease compared with non-laboratory staff (relative risk 21.5, 95% CI 4.5–102.5).<sup>26</sup>

Laboratories performing mycobacterial drug susceptibility testing must meet the requirements of a PC3 facility.<sup>24</sup>

### Susceptibility testing for *Mycobacterium tuberculosis*

The DSTs must be performed using a liquid culture system so that results are available promptly. Using these methods, laboratories should aim to report MTBC DST results within an average of 15–30 days from the time of the original specimen reception.<sup>9</sup> The DSTs themselves can generally be completed within 7–21 days of obtaining the initial *M. tuberculosis* isolate from the primary cultures.<sup>9,16</sup>

Initial susceptibility testing should include isoniazid (high- and low-level concentrations as appropriate), rifampicin, ethambutol and pyrazinamide. The critical concentrations for these antibiotics are listed in Table 4.<sup>16</sup> An expert group convened by the WHO TB Programme has recently provided updated recommendations for critical concentrations for second-line anti-TB drugs, including interim guidance for delamanid and bedaquiline (table 4).<sup>62</sup> This group reviewed the distribution of minimum inhibitory concentrations (MICs), sequence data and clinical breakpoints for the aforementioned antibiotics. Critical concentrations (CCs) and clinical breakpoints (CBs) were revised or newly established. A CC is defined as the minimum concentration of an antibiotic that suppresses

the growth of 99% of phenotypically-wild-type strains of the *M. tuberculosis* complex. The CB is defined as a minimum inhibitory concentration (MIC) above the CC that separates strains that are likely to respond to treatment from strains that are unlikely to respond to treatment. The CB is determined based on clinical outcome data and pharmacokinetic/pharmacodynamics (PK/PD) principles, including increased dosing.

For isolates demonstrating isoniazid resistance at the CC but susceptible at the higher concentration, US authorities have previously recommend adding the following comment to the report: ‘These test results indicate low-level resistance to isoniazid. Some experts believe that patients infected with strains exhibiting this level of INH resistance may benefit from continuing therapy with INH. A specialist in the treatment of tuberculosis should be consulted regarding the appropriate therapeutic regimen and dosages’.<sup>63,64</sup> Australian laboratories could consider adding a similar comment in these circumstances after discussion with their clinical TB specialists.

WHO recommends for laboratories to perform susceptibility testing for the fluoroquinolone used in their country and to phase out ofloxacin testing.<sup>62</sup> Moxifloxacin should therefore be tested in Australia. A CC and a CB are provided for moxifloxacin susceptibility testing (Table 4). Resistance at the lower breakpoint is associated with resistance to earlier-generation fluoroquinolones and with the presence of *gyrA* mutations.<sup>65,66</sup> The clinical breakpoint assumes high-dose moxifloxacin treatment at 800 mg daily. If ofloxacin testing is performed, patients with ofloxacin resistance but ‘low-level’ moxifloxacin resistance have been shown to benefit from high-dose moxifloxacin treatment. The use of moxifloxacin in such patients should be used cautiously as a companion drug and not as a key element in the patient’s core regimen.

Drug susceptibility tests must be performed in the following circumstances:<sup>16,63</sup>

- all initial isolates of *M. tuberculosis*;
- isolates from patients who remain culture-positive after 3 months of treatment;
- isolates from patients who are clinically failing treatment; or
- an initial isolate from a patient relapsing after previously successful TB treatment.

Any isolate with rifampicin resistance detected by a rapid molecular method must have second-line DST performed in parallel with first-line DST. At a minimum, second-line testing should include amikacin, capreomycin (optionally kanamycin) and a fluoroquinolone (not ciprofloxacin).

**Table 4: MGIT 960 critical concentrations for first- and second-line drug susceptibility testing of *Mycobacterium tuberculosis***<sup>16,62</sup>

Drug group	Drug	DST critical concentration <sup>b</sup> µg/ml
First-line oral anti-TB agents	Isoniazid (low, high) Rifampicin Ethambutol <sup>c</sup> Pyrazinamide <sup>c</sup>	0.1, 0.4 0.5 5.0 100
Second-line agents – medicines for longer course therapy of MDR-TB grouped as per WHO		
Group A	Moxifloxacin or Levofloxacin Bedaquiline <sup>e</sup> Linezolid	0.25 (CC) / 1.0 (CB) <sup>d</sup> 1.0 1.0 1.0
Group B	Clofazimine <sup>e</sup> Cycloserine <sup>f</sup>	1.0 NA
Group C Other second-line anti-TB agents (used when a WHO approved regimen cannot be constructed from group A and B drugs or as specified for the shorter course MDR-TB regimen)	Ethionamide Prothionamide Amikacin Streptomycin <sup>g</sup> <i>Para</i> -aminosalicylic acid (PAS) <sup>f</sup> Delamanid <sup>e</sup> Imipenem-cilastatin or meropenem (carbapenems) <sup>f</sup>	5.0 2.5 1.0 1.0 NA 0.06 NA
Agents where WHO no longer recommends use in MDR-TB treatment regimens (DST to be phased out)	Ofloxacin Kanamycin Capreomycin	2.0 2.5 2.5

a This is an updated tabulation of critical concentrations, effective 29 April 2021. See Appendix A for clarification.

b NA: not applicable.

c Ethambutol and pyrazinamide can be used as Group C drugs when treating MDR-TB.

d Critical concentration (CC), clinical breakpoint (CB).

e Critical concentrations for bedaquiline, clofazimine and delamanid are interim WHO recommendations.

f No critical concentration is recommended for cycloserine, PAS or carbapenem class as there is insufficient data to set one.

g Streptomycin susceptibility testing is considered optional due to limited availability in Australia; the agent may be considered for use in drug resistant (DR)-TB if amikacin can't be used (unavailable or documented resistance).

Second-line drug susceptibility tests should be performed also on:

- all MDRTB isolates (i.e. isolates demonstrating isoniazid and rifampicin resistance);
- all isolates demonstrating resistance to  $\geq 2$  first-line drugs; and
- isolates from patients experiencing severe adverse reactions to first-line agents.

Isolates demonstrating isoniazid resistance should be tested for fluoroquinolone susceptibility (at least) where a fluoroquinolone containing regimen is planned.

Before reporting a DST result, it is most important to exclude contamination in any drug tube that shows resistance. This can be done by inspecting the tubes visually for micro-colonies, confirming the absence of turbidity (which indicates non-MBTC contamination), and performing a ZN-stain looking for cording AFB. Exclusion of contamination as a cause of false resistance is especially important when reporting a putative MDRTB or XDRTB case. The following checks should be performed:

- the above macroscopic and microscopic checks for contamination; and
- inoculation of a blood agar plate to detect bacterial contamination; and
- a test to detect *rpoB* gene mutations as supporting molecular evidence of at least rifampicin resistance; and
- a Hain second-line (MTBDRs<sub>l</sub>) assay as supporting molecular evidence of resistance to second-line injectable agents and quinolones; and most importantly
- inclusion in the second-line drug susceptibility testing of an additional tube containing 500 ug/ml of *p*-nitrobenzoic acid (PNB) to detect mixed cultures with NTM. Growth of *M. tuberculosis* complex is inhibited by PNB, while almost all NTM are resistant.<sup>67</sup>

## Clarithromycin susceptibility testing for the *Mycobacterium avium* complex

Macrolides (azithromycin and clarithromycin) are the only antimicrobial agents where a correlation between *in vitro* susceptibility and clinical response for MAC has been demonstrated.<sup>68</sup> The mechanism of mutational resistance in MAC isolates is the same for clarithromycin and azithromycin, however susceptibility testing with clarithromycin is recommended due to the poor solubility of azithromycin. Susceptibility should be performed using broth-based microdilution or macrodilution and is recommended in the following circumstances:

- Baseline isolates from significant MAC infections may also be tested (or stored and tested retrospectively if the patient does not respond to treatment).
- Clinically significant isolate from a patient who has received previous macrolide therapy (i.e. clarithromycin or azithromycin);
- patients who have developed MAC bacteraemia whilst on macrolide preventative therapy, typically in the context of HIV/AIDS;
- patients failing or relapsing on macrolide therapy; and
- DST should be repeated after 3 months of treatment (for patients with disseminated disease) or 6 months (for patients with pulmonary disease) if the culture is still positive or the patient shows no clinical improvement.<sup>16</sup>

The methodology and interpretative breakpoint are described in the relevant document (M24-A2) published by CLSI.<sup>16</sup> At the current time EUCAST does not have recommendations for clarithromycin susceptibility testing of NTMs including MAC.

**Table 5: Broth microdilution interpretative criteria for rapid-growing mycobacteria<sup>16</sup>**

Agent	Susceptible	Intermediate	Resistant
	Minimum inhibitory concentration (µg/ml) for category		
Amikacin	≤ 16	32	≥ 64
Cefoxitin	≤ 16	32–64	≥ 128
Ciprofloxacin	≤ 1	2	≥ 4
Clarithromycin <sup>a</sup>	≤ 2	4	≥ 8
Doxycycline	≤ 1	2–4	≥ 8
Imipenem	≤ 4	8–16	≥ 32
Linezolid	≤ 8	16	≥ 32
Meropenem	≤ 4	8–16	≥ 32
Moxifloxacin	≤ 1	2	≥ 4
Trimethoprim-sulphamethoxazole	≤ 2/38	—	≥ 4/76
Tobramycin	≤ 2	4	≥ 8

a The final reading of the clarithromycin result should be delayed to day 14 to detect the inducible macrolide resistance. Users should refer to the original CLSI document for other footnotes related to this table.<sup>16</sup>

### Susceptibility testing of *Mycobacterium kansasii*

Drugs which are clinically active against *M. kansasii* are rifampicin, isoniazid, ethambutol and clarithromycin. Treatment failure is associated with rifampicin resistance and therefore testing of rifampicin and clarithromycin only is recommended in the following circumstances:

- All clinically relevant initial isolates of *M. kansasii*
- For patients failing or relapsing on treatment

The methodology and interpretative breakpoints are described in the relevant CLSI document M24-A2.<sup>16</sup>

### Susceptibility testing of rapidly growing non-tuberculous mycobacteria

Using broth microdilution, all clinically relevant RGM should be tested against the following: amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, imipenem, linezolid, co-

trimoxazole and tobramycin.<sup>16,57</sup> Broth microdilution is technically demanding and interpretation of end points requires a substantial level of experience. Automated reading and data interpretation may be utilised to streamline broth microdilution DST. Laboratories which isolate a clinically relevant RGM should refer isolates to an MRL. Alternatively, non-MRL laboratories may perform RGM DST with suitable NATA accreditation and satisfactory participation in external QAP.

The broth microdilution methodology and interpretative breakpoints are described in the relevant CLSI document M24-A2.<sup>16</sup> Notably, the final reading of the clarithromycin result should be delayed to day 14 to detect the inducible macrolide resistance described above. Table 5 reproduces the critical breakpoints recommended in the CLSI document.<sup>16</sup>

Laboratories and clinicians should recognise that the RGM breakpoints have not been set using modern approaches integrating knowledge about wild-type distributions of MICs, PK/PD assessments, and clinical outcome data.<sup>69</sup>

The clinical utility of these results are therefore uncertain. The US-European CF consensus document nicely summarises the situation, “Antibiotic choices should be guided but not dictated by drug susceptibility testing.”<sup>57</sup>

Susceptibility testing for other NTM may be performed following close communication between the treating clinician, the primary mycobacterial culture laboratory, and the relevant MRL/DST laboratory. Published guidelines on NTM DST should be followed in all circumstances.<sup>16</sup>

## Guidelines for Nucleic Acid Amplification Tests

### General requirements for a Microbiology Nucleic Acid Amplification Facility

In 2013, NPAAC published updated standards and guidelines for laboratories performing NAAT for biological agents.<sup>20</sup> The NPAAC document addresses specimen collection, transportation, reagent preparation, nucleic acid extraction, amplification, product detection, data recording, reporting, sample storage and quality assurance. Laboratories performing NAAT for TB diagnosis must comply with these NPAAC recommendations. Some of the standards and guidelines of particular relevance to TB NAAT are highlighted below.

1. Samples that have been used for other tests prior to NAAT are at increased risk of cross-contamination. Wherever possible, NAAT should be performed on dedicated samples or on aliquots taken before other tests are performed.
2. NAATs are capable of detecting very small quantities of nucleic acid and are therefore liable to false-positive results due to contamination events. Staff competence, laboratory design and routine use of controls limit and detect these contamination events. Three physically-separated areas are required in a NAAT laboratory for: DNA extraction, reagent preparation, and amplification/product detection. The movement of specimens and equipment shall be unidirectional from pre- to post-amplification areas. At least one negative control and a weak positive control must be subject to the whole test process including DNA extraction.

Implementation of the legislation regarding IVDs implemented by TGA will also impact on laboratories performing TB NAAT using either commercial or in-house assays.<sup>21</sup> As *M. tuberculosis* represents a moderate public health risk, a personal risk, and is included on the NNDSS, TB diagnostics are classified as class 3 IVDs.<sup>22</sup>

All commercially-supplied IVDs were required to be included in the Australian Register of Therapeutic Goods (ARTG) unless otherwise exempt by 30 June 2015. Laboratories with class 1–3 in-house IVDs are exempt from the requirement to include them in the ARTG providing the laboratory is accredited by NATA to either ISO15189 or ISO 7025, meet the NPAAC standard, Requirements for the development and use of in vitro diagnostic medical devices, and provide a notification to the TGA of the class 1–3 in-house IVDs before 1 July 2017. Laboratories are referred to the relevant documentation on the TGA website (<https://www.tga.gov.au/ivd-guidance-documents>).

### Basic principles about tuberculosis NAAT testing

The original 2006 NTAC guidelines listed some basic principles regarding TB NAAT.<sup>3</sup> The earlier section summarising recent advances in TB molecular tests highlighted that GeneXpert and other TB NAATs still lacked sensitivity relative to culture, particularly in smear-negative specimens (table 1). For example, GeneXpert has a sensitivity of 57–83% in smear-negative samples (which is comparable with or better than the performance of other commercial and in-house NAAT), and has an estimated limit of detection of 131 (95% CI: 106–176) colony forming units (CFU) per millilitre of sputum (compared with high-quality culture that can detect approximately 10 CFU/ml).<sup>29</sup> The basic principles regarding TB NAAT therefore remain relevant and are restated.

1. NAAT is a supplemental test and does not replace smear microscopy or mycobacterial culture.
2. NAAT should not be performed automatically on every TB specimen or TB suspect.
3. As with all mycobacterial investigations, the decision to perform NAAT and the result interpretation requires close liaison between the clinician and laboratory staff.

4. Clinical material (eg. CSF) should not be preserved for NAAT if this compromises the ability to perform established tests of better diagnostic utility (eg. culture).

NAAT on non-respiratory specimens has not been approved by the FDA. On the other hand, WHO has supported the use of Gene Xpert for testing specific extrapulmonary specimens such as CSF and lymph node specimens.<sup>31</sup>

### Indications for performing nucleic acid amplification tests for tuberculosis diagnosis

Despite these limitations in TB NAAT, the US CDC issued updated guidelines in 2009, stating that ‘NAA testing should be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary TB for whom a diagnosis of TB is being considered but has not yet been established, and for whom the test result would alter case management of TB control activities’.<sup>70</sup>

In contrast to these aggressive US recommendations, the National Institute for Health and Care Excellence (NICE) in the United Kingdom (UK) limited NAAT testing on primary specimens to specific circumstances:

- ‘if rapid information about mycobacterial species would alter the person’s care’; or
- before conducting a large contact-tracing initiative; or
- the patient is HIV-positive or a child; or
- for rapid rifampicin-resistance detection in an MDRTB suspect; or
- on smear-positive biopsy material that has been placed mistakenly in formalin.<sup>13</sup>

The Canadian guidelines have similarly restricted NAAT testing to:

- all new smear-positive cases;

- in smear-negative patients upon request by the physician or the TB control program.<sup>10</sup>

Cost-effectiveness analyses have supported a targeted approach to TB NAAT. For example, Hughes et al. performed a cost-effectiveness analysis of various diagnostic strategies involving smear microscopy, NAAT and culture.<sup>71</sup> The cost-effective strategy at a threshold of £20,000 per quality adjusted life year (QALY) was smear microscopy followed by culture routinely. A full work-up of microscopy, NAAT and culture became cost-effective as the TB prevalence increased (ie. in patients strongly suspected of TB).

### Developing an algorithm for NAAT testing of respiratory specimens

Each mycobacteriology laboratory will need to develop a NAAT testing algorithm based on the above principles but also considering the characteristics of their patient population, the prevalence of TB and NTM cases in their locale, the potential sample load, the performance characteristics of their TB NAAT, and their laboratory size and resources.

The following indications gleaned from the international literature should be considered for inclusion in an algorithm:<sup>10,13,32</sup>

- respiratory smear-positive specimens (because detection of an *rpoB* mutation by the Xpert MTB/RIF test or similar assay provides an early indication of MDRTB and a negative result strongly suggests NTM infection);
- respiratory smear-negative specimens from a patient with a high probability of TB, when prompt management and public health decisions are required;
- respiratory specimens from HIV-positive patients if TB is reasonably suspected clinically;
- respiratory specimens from children if TB is reasonably suspected clinically;

- smear-positive biopsy material that has been placed mistakenly in formalin and no fresh specimen is available for culture (if the NAAT has been validated for this purpose);
- selected non-respiratory specimens where an urgent management decision is necessary (recognising that such tests have not been validated or approved, and there is currently very low quality evidence available);
- the Xpert MTB/RIF Ultra assay has outperformed conventional Xpert and TB culture for diagnosis of TB meningitis and should be considered as the preferred initial investigation for TBM;<sup>75</sup> and
- inform decision making about lifting AP for patients with suspected pulmonary TB by obtaining negative Xpert MTB/RIF results on 1–2 respiratory specimens (another NAAT may be used if properly validated for this purpose).

The use of NAAT is considered inappropriate when a patient is respiratory smear-negative and has a low probability of TB. Close clinical and laboratory liaison is required before deciding to perform NAAT on a non-respiratory specimen, particularly a paucibacillary non-respiratory specimens (eg. pleural fluid, ascitic fluid), and when interpreting the result.

## Staff Screening and Health Care

Safety in the laboratory is the responsibility of management, the biosafety committee (BC), appointed safety officers, the laboratory supervisor, and the laboratory personnel. The Australian/New Zealand Standard 2243.3: 2010 and the European Committee for Standardization biorisk management document CWA 15793 describe the responsibilities of each of these groups.<sup>24,25</sup>

Personnel working in mycobacteriology laboratories require:

1. thorough initial training in TB laboratory procedures and safety measures;
2. ongoing education; and
3. additional health checks.

All new TB laboratory staff should have a two-step tuberculin skin test (TST) performed. The NTAC position statement on interferon  $\gamma$ -release assays (IGRAs) explains that the choice of test for serial testing in healthcare workers, including laboratory staff, is controversial.<sup>72</sup> A preference remains for TST for serial testing because IGRAs have been bedevilled by higher rates of reversions and conversions when used for serial testing.<sup>72</sup> An initial positive TST result must be followed-up by chest X-ray (CXR) and a medical consultation. TST-negative staff members should be required to have annual skin tests; any TST conversion must be followed by CXR, medical examination, and consideration of chemoprophylaxis. Similar investigations should be instituted following a laboratory accident or known exposure event.

These annual screenings may fortuitously detect a recent TB infection. It is far more important that laboratory personnel are educated about the risks of TB, the likely presenting symptoms (eg. chronic cough, weight loss, fever), and the need to inform their treating doctor that they work in a TB laboratory.

Laboratory personnel must also be informed of the medical conditions that increase the risk of progression to active TB disease (ie. HIV infection, organ transplantation, steroid use, malignancy, chronic renal failure, diabetes). Personnel with these conditions can then be encouraged to discuss their situation with their treating physician and laboratory administration, and to consider an alternative work environment within the microbiology laboratory.

Finally, the Australian/New Zealand Standard 2243.3: 2010 suggests that BCG vaccination 'should be considered for staff with high risk of exposure to TB and as recommended by State/Territory TB control authorities'.<sup>24</sup> The efficacy of BCG remains controversial with reported protection levels varying between 0–80%.<sup>73</sup> Efficacy of vaccination in adulthood is even more controversial. Despite these uncertainties, interest in BCG vaccination has increased with the advent of MDRTB. BCG vaccination has negligible side effects and may provide some protection irrespective of the drug susceptibility status of the infecting strain. However, BCG vaccination confounds the alternative strategy of performing regular TSTs on HCWs and offering preventative therapy to 'converters'. Many HCWs do not comply with TST screening and preventative therapy for MDRTB-exposed individuals is problematic. In these uncertain circumstances, the following recommendations seem reasonable:

1. No benefit is to be gained from re-vaccinating laboratory personnel who have received BCG previously. This recommendation is true irrespective of the person's TST status.
2. Laboratory personnel should be required to participate in a TST and health screening program.
3. Non-vaccinated laboratory personnel at increased risk of MDRTB exposure (eg. those working in laboratories performing DSTs) should be offered BCG after counselling about the advantages and disadvantages of the vaccination.

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## Appendix A

### Amendment 1: effective 29 April 2021

The World Health Organization (WHO) has lowered the recommended critical concentration for rifampicin in Mycobacteria Growth Indicator Tube (MGIT) by one dilution to 0.5 ug/ml (previously 1.0 ug/ml)<sup>1</sup> for drug susceptibility testing (DST) of *Mycobacterium tuberculosis*.

WHO has also revised recommended treatment regimens for multidrug-resistant tuberculosis (MDR-TB), including a revised grouping of second-line antimicrobial agents for the longer treatment regimen for MDR-TB.<sup>2</sup>

The National Tuberculosis Advisory Committee's *Revised Guidelines for Australian laboratories performing mycobacteriology testing* has been updated in line with this advice (online edition only). Accordingly, **Table 4** of the Revised Guidelines has been amended.

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