Guidelines for Australian Mycobacteriology Laboratories

National Tuberculosis Advisory Committee

Executive summary

Guidelines for Australian laboratories performing tuberculosis (TB) microscopy and culture have been developed through extensive consultation with expert groups. The aims of these guidelines are:

1. to provide consensus recommendations on the infrastructure, equipment and work practices required by mycobacteriology laboratories;
2. to inform laboratory administrations and governments of the necessary level of investment required to maintain modern mycobacteriology facilities; and
3. to provide informal measures for reviewers inspecting mycobacteriology facilities.

These guidelines include safety recommendations based largely on the Australian/New Zealand Standard 2243.3 *Safety in laboratories – Microbiological aspects and containment facilities.* However, these standards have been rationalised and PC2 facilities with additional processes and precautions in place are recommended for the majority of TB investigations. Guidelines are also provided on staff training, education, health screening and vaccination. Certain procedures and work practices are recommended for mycobacteriology laboratories to guarantee safety, high-quality results, and prompt turnaround times. These guidelines will be reviewed each 1–2 years and feedback from expert groups and individuals is welcomed.

Introduction

Tuberculosis (TB) represents an increasing threat to global health with at least 2 million deaths and 8 million new cases annually. Unlike the United States of America (USA) and some European countries where TB epidemics again occurred in the late 1980s–1990s, Australia has maintained a very low incidence of TB with just 1,159 active cases reported in 1999 (i.e. 6.1 cases per 100,000 population).[1] Australia has maintained these low rates despite migration from TB–endemic countries, because of the continued efforts of clinical, laboratory and public health personnel involved in TB control. However, Australia must continue these efforts and must also support TB control efforts in our region because over 60 per cent of the global TB burden occurs in South East Asia and the Pacific.

Following the USA TB epidemic in the early 1990s, the US Centers for Disease Control and Prevention (CDC) set demanding criteria for mycobacteriology laboratory performance:[2,3]

- acid-fast examinations to be reported within 24 hours of specimen+ collection;
- identification of *Mycobacterium tuberculosis* complex (MTBC) within an average of 10–14 days; and
- reporting of drug susceptibility results within an average of 15–30 days.

These 'rapid' turnaround times (TATs) can only be met by increasing investment in mycobacteriology staff and by the introduction of new expensive broth-based culture systems. A 1991 review of USA laboratory practices revealed that only 29 per cent and 20 per cent were using the BACTEC radiometric system for culture and susceptibility testing respectively, resulting in substantial delays in the reporting of results.[4] A 1994 follow-up review of laboratory practices found a marked improvement in performance with laboratories reporting microscopy results within 24 hours having risen from 52.1 to 77.6 per cent, the reporting of MTBC within 21 days increasing by 22.1 per cent to 72.9 per cent, and susceptibility testing being completed within 28 days rising from 16.7 to 48.9 per cent.[5] These results were associated with the introduction of new technologies. The percentage of laboratories using fluorescence microscopy, BACTEC for primary culture, and BACTEC for susceptibility testing having risen from 27.1 to 79.6 per cent, 74.5 to 100 per cent, and 26.2 to 73.3 per cent respectively.[5]

Mycobacteriology laboratory services in Australia

Despite having low incidences of TB in Australia and other industrialised countries, their mycobacteriology laboratories are increasingly expected to meet the USA standards. Australian laboratories have reported 700–760 isolates of *M. tuberculosis* complex per year between 1996–1999.[6] Only approximately 75–85 Australian laboratories perform TB cultures and the majority of this work is performed

in the five Mycobacterium Reference Laboratories (MRLs) and other large centres. Smaller laboratories therefore may not have had the necessary workload to maintain expertise or to justify investment in the new technologies.

These problems are perhaps best expressed in the results of the Royal College of Pathologists of Australasia (RCPA) national quality assurance program (QAP). Since 1996, smears containing between 1–9 acid-fast bacilli (AFB)/high power field (i.e. 2+) have been called negative by 1.5–6.0 per cent of laboratories. Of equivalent concern is that a similar number of laboratories (2.4–5%) are reporting false-positive results for QAP smears negative for AFB.

Of the 80 or so laboratories performing culture, up to 16 per cent of laboratories have failed to recover MTBC from QAP samples that on occasion have contained +++ AFB. The majority of culture errors have occurred in laboratories which process fewer than 1,000 specimens per year. In a 1999 item (RCPA 99:6:3), two samples were sent for mycobacterial culture. For the sample containing approximately 500 colony forming units of *M. bovis* (BCG)/sample, 80/83 (96%) of laboratories successfully isolated mycobacteria, but for the other sample containing approximately 50 colony forming units BCG/sample, only 63/83 (76%) were able to recover mycobacteria. Three of 83 laboratories failed to recover mycobacteria from either sample. These results suggest that a minority of laboratories are failing in one or more areas of specimen processing, media quality, culture performance, or culture interpretation. Additionally, the number of false-positive reports has risen from 0 per cent in the 1995 and 1996 RCPA-QAP to a high of 5.3 per cent in 1999. Although the number of laboratories performing culture has remained steady, there is an apparent 'ebb and flow' with some laboratories ceasing to perform culture whilst others have begun to do so.

Rationale for national guidelines

These guidelines have been produced to assist several groups directly and indirectly involved in maintaining the high quality of mycobacteriology investigations performed in Australia.

Consensus guidelines for a mycobacteriology laboratory

These guidelines aim to document the infrastructure, equipment, staffing and work practice requirements for a modern mycobacteriology laboratory. These guidelines have been developed through extensive consultation with the Mycobacterium Reference Laboratories, the Mycobacterium Special Interest Group (SIG) of the Australian Society for Microbiology (ASM), the Public Health Laboratory Network (PHLN), the Royal College of Pathologists of Australasia (RCPA), and other interested parties. Mycobacteriology laboratory staff can therefore use these guidelines as a benchmark tool for assessing their own laboratory performance.

Laboratory administration

Laboratories must balance the increasing investment required to provide a modern high-quality mycobacteriology service against the expected income from a limited number of TB and other mycobacteriology requests. 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 costs of providing these services.

Laboratory reviewers

The reviewers for the National Association of Testing Authorities (NATA)/RCPA often do not have extensive expertise in mycobacteriology. These guidelines aim to provide 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. For example, a high-quality laboratory may fulfil all requirements but may not have an 'adequate' workload to maintain expertise. Such a laboratory could adequately compensate by additional training for staff and demonstrated close liaison with the relevant MRL or other major laboratory. In contrast, an inadequate laboratory is likely to be deficient in several aspects.

Government authorities and the general public

Australia has one of the lowest TB rates in the world. However, continued funding is required to maintain this enviable position. The experience in New York City in the 1980s–1990s demonstrates the alternative outcome.[7] Reduced TB funding in the late 1970s–early 1980s resulted in degraded TB services including a deterioration in the TB laboratory system. An epidemic followed with more than 20,000 excess cases including outbreaks of multidrug-resistant disease. Failure of the under-resourced laboratories to detect TB promptly and to perform drug susceptibility tests (DST) expeditiously were contributing factors to this TB epidemic.[2,7] Over US$1 billion has been spent bringing TB back under control in New York City.[7] Some of these funds have been spent renovating and improving the TB laboratories.

This document therefore 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 are continuing to be provided throughout Australia.

Formulation of the TB laboratory guidelines

These guidelines reaffirm and reiterate the biosafety requirements for Australian mycobacteriology laboratories as outlined in the latest Australian/New Zealand Standard 2243.3 *Safety in laboratories – Microbiological aspects and containment facilities.*[8] Laboratories must also comply with the National Pathology Accreditation Advisory Council (NPAAC) Standards for Pathology Laboratories[9] and relevant NATA requirements.

The guidelines specific for TB laboratories have been developed through extensive consultation with MRL staff, the ASM Mycobacterium SIG, PHLN members, RCPA, and other interested parties. Similar guidelines for USA laboratories have also been referenced.[2,3] The New Zealand Ministry of Health have published a large compendium entitled *Guidelines for tuberculosis control in New Zealand,* which includes a chapter for the mycobacteriology laboratory (available from: http://www.moh.govt.nz/49ba80c00757b8804c25667300d47d0/4760df3580a6f5b5cc256c86006ed394?OpenDocument). There are no matching documents produced by European or British authorities (FA Drobniewski, PHLS Mycobacterium Reference Unit, King's College Hospital, London, personal communication). These Australian guidelines will be reviewed each 1–2 years and feedback from expert groups is welcomed.

Risk group classification of Mycobacterium tuberculosis

Australian/New Zealand Standard 2243.3 *Safety in laboratories – Microbiological aspects and containment facilities* recognises *M. tuberculosis* as a Risk Group 2 organism; multidrug-resistant strains are considered to be Risk Group 3 organisms. This classification leaves mycobacteriology laboratories with an interesting dilemma. Clinical specimens will be processed, cultures performed, and susceptibility tests completed before laboratory staff will know whether they should have undertaken these tests in a Physical Containment Level 2 (PC2) or PC3 facility.

One approach would be to treat all specimens as containing MDRTB organisms until proven otherwise and hence require all TB investigations be performed in a PC3 facility. While providing the highest level of staff protection, this approach is arguably excessive because Australia has a very low rate of MDRTB (0.5–0.9% in 1998–1999,[6]) and only 6–7 laboratories are undertaking DSTs. Many Australian mycobacteriology laboratories do not meet all of the requirements of a PC3 facility. Mandatory use of PC3 facilities would therefore require major infrastructure investment or would result in an excessive workload concentrating on the limited number of PC3 laboratories.

A reasonable compromise position could be for all mycobacteriology investigations to be undertaken in PC2 facilities with additional processes and precautions in place (see below). Laboratories undertaking more than 5,000 cultures per year, performing DSTs, or knowingly handling MDRTB strains should have PC3 facilities or have building plans to acquire PC3 facilities by 2007.

Guidelines for a laboratory performing smear microscopy

Approximately 80 laboratories in Australia perform smear microscopy then forward the specimen to another laboratory for mycobacterial culture. The microscopy-only laboratories almost universally perform direct Ziehl-Neelsen (ZN) smears. While opening sputum containers and making smears may produce aerosols, there is no epidemiological evidence in low-income countries associating smear preparation with any measurable increased risk of acquiring TB infection. The World Health Organization (WHO) and the International Union Against TB and Lung Disease (IUATLD) therefore consider smear preparation a low-risk procedure.[10] 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.[8] The following (additional) requirements must be emphasised:

1. The smear preparation procedure must be performed in a Class I or Class II biosafety cabinet (BSC).
2. The operator must wear gloves and a long-sleeved gown.
3. Any manipulation involving shaking, mixing or sonication must be performed in the BSC and a period of at least 5 minutes elapse before the container is opened in the BSC.

4. In the unlikely event that a concentrated smear is being prepared, a centrifuge with sealed rotors or safety cups must be used and must be capable of attaining 3,000 *g*.

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 (IATA) regulations (summarised in AS/NZS 2243.3,[8]). A specific Australian standard on the shipment of biological materials is also in preparation.

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. On weekends, the requesting doctor should discuss urgent requests for TB smear microscopy with the clinical microbiologist. 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 such weekend specimens are urgent or non-urgent.

2. Specimens for cultures should be transported to the relevant laboratory within 24 hours.

3. A positive- and a negative-control smear should be included with each batch of smears.

4. Positive results should be quantified using the IUATLD/WHO scale:[10]

negative	No acid-fast bacilli (AFB) in at least 100 high power fields (HPFs)
exact figure/100	1–9 AFB per 100 HPFs
+	10–99 AFB per 100 HPFs
++	1–10 AFB per field in at least 50 HPFs
+++	More than 10 AFB per field in at least 20 HPFs

5. 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 20 ZN smears per day on average. More smears (2–3-fold) can be read per day if a fluorochrome stain is used.

6. 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.

7. 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.

8. Larger laboratories that process many specimens (and perform cultures) may use a fluorochrome stain. All fluorochrome-positive slides from new smear-positive patients must be checked by ZN stain.

9. The laboratory should have the ability to monitor the number of specimens collected per year, the number of patients from whom 1, 2 or 3 sputa are collected, and the number of smear-positive results in TB suspects and patients on treatment.

10. The laboratory must participate in an external quality assurance program. The RCPA program sends 8–10 AFB smears per year. Quantitation errors are of minor significance.[10] Similarly, scanty false-negative results are understandable if the QAP sends a slide with 1–9 AFB/100 fields. In contrast, >1 false-positive or high false-negative result in any one year should trigger remedial action.

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.[10]

1. The 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 (e.g. refrigeration at 4° C).

2. Appropriate containers should be provided to the patient.

3. Sputum specimens should be collected in well-ventilated areas away from other patients. Patients at high-risk of having TB should be referred to hospitals where they can be evaluated and managed appropriately.

4. For patients at high-risk of having TB, sputum collection and cough-inducing procedures should be performed in negative-pressure ventilation rooms. Health-care workers (HCWs) should wear respiratory protection (i.e. a properly fit-tested high-efficiency N95-standard mask or powered air-purifying respirator – PAPR) when present in rooms or enclosures in which cough-inducing procedures are being performed on patients who may have infectious TB. These high-risk patients should also be managed appropriately before and after the specimen collection to limit cross-infection to other patients and to HCWs. The US Centers for Disease Control and Prevention have released extensive guidelines on reducing TB transmission in the health-care setting.[11]

Guidelines for laboratories performing mycobacterial cultures

As discussed earlier, 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. Alternatively, these laboratories could be considered PC3 laboratories without some of the building and engineering requirements. Nonetheless, laboratories undertaking more than 5,000 cultures per year, performing susceptibility tests, or knowingly handling MDRTB strains should have PC3 facilities or have building plans to acquire PC3 facilities by 2007.

General laboratory facilities, equipment and work practices

1. The TB culture laboratory must be in a self-contained room physically separated from other areas.

2. Access to the TB laboratory must be limited to staff trained to work in the area. Access should be restricted by lockable doors.

3. A pressure steam steriliser must be available for decontaminating laboratory waste, preferably within the laboratory.

4. A directional air flow shall be maintained by extracting room air. Recirculation is permitted but not into areas outside the PC2-PC3 facility.

5. All procedures must be performed in a Class I or Class II biosafety cabinet.

6. The operator must wear gloves and a long-sleeved gown. These personal protection items must not be worn outside of the TB laboratory. N-95 HEPA masks should be provided for staff to clean laboratory spills or if other accidents occur.

7. Any manipulation involving shaking, mixing or sonication must be performed in the BSC and a period of at least 5 minutes elapse before the container is opened in the BSC.

8. A centrifuge with sealed rotors or safety cups must be used. This centrifuge should attain 3,000 *g* to reliably sediment AFB.

Requirements specific to a laboratory performing TB culture

1. A scientist with a university degree (or equivalent training and experience) should be responsible for the TB laboratory. All staff working in the TB laboratory should have been suitably trained and have evidence of on-going 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. A TB culture facility should process 20 or more 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, any smear-positive specimen should be cultured on the day of smear preparation.

4. All specimens should be inoculated in a broth-based culture system +/– onto solid media.

   The BACTEC and newer non-radiometric systems have revolutionised mycobacteriology providing TATs appreciably faster than those achieved by culture on solid media.[2–5,12] Broth-based culture systems should therefore be used by default. USA authorities recommend that each specimen should also be inoculated onto solid media to detect strains that may not grow in broth. 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. Processing of multiple specimens from each TB suspect also increases the sensitivity of culture. Furthermore, the requirement for solid media adds to the cost and workload of a laboratory. Selective use of solid media may therefore be acceptable (e.g. on all sterile site specimens such as tissues or CSF, and on any smear-positive specimen).

   Specimens from skin, lymph nodes and abscesses that may contain pathogenic non-tuberculous mycobacteria (NTM) should also be inoculated onto/into additional media for incubation at 30° C.

5. Specimens from sterile sites not requiring decontamination (e.g. cerebrospinal fluid, biopsies) should be directly inoculated into the broth- and solid-media systems.

6. 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 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 and recovery rates of NTM is far more important.

7. Contamination rates should be recorded. Bacterial contamination rates less than 8 per cent are acceptable representing the best balance between excessive contamination and overly stringent decontamination (that risks false-negative culture results).

8. Laboratories must be alert to cross-contamination between specimens resulting in false-positive results. Laboratory cross-contamination should be considered in the following circumstances:[13]

    - 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 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 are inoculated with the same specimen.

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

    - reviewing the laboratory logbook for other culture-positive specimens processed at the same time;
    - reviewing the patient's history, radiological investigations, clinical course, and response to therapy; and
    - genotyping of the suspicious isolates which may demonstrate identical profiles to laboratory control strains (e.g. H37Rv) or to isolates from epidemiologically-unrelated patients processed on the same day;
    - reviewing the laboratory procedures.

9. Non-automated broth-based cultures should be read every 2–3 days for weeks 1–3, and weekly thereafter for 6–12 weeks (depending on the specimen type and smear result). Solid media should be read twice weekly for weeks 1–4 then weekly thereafter.

10. 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). The initial mycobacterial isolate from a patient must be identified as *M. tuberculosis* complex, *M. avium* complex, or another NTM. The indications for performing further susceptibility testing on *M. tuberculosis* isolates are listed below and the indication for referring NTM are provided in the section headed 'Referral of non-tuberculosis mycobacteria cultures'.

11. Laboratories performing cultures but referring isolates for identification and DST must send positive cultures to the reference laboratory within 48 hours of culture positivity. The isolate must be accompanied by documentation of all relevant clinical and laboratory information (e.g. patient details, original specimen type, AFB smear result, associated histological investigations that may have been performed on the same specimen).

12. A rapid (molecular) test is preferred for confirming growth of MTBC in ZN-positive cultures. The AccuProbe nucleic acid hybridisation test (Gen Probe, San Diego, CA) or an MTBC-specific nucleic acid amplification test (NAAT) are recommended. HPLC may be a reasonable alternative in laboratories with this equipment and expertise. The BACTEC NAP test is no longer considered acceptable because mixed cultures, growth temperatures and phases, and certain NTM can all produce anomalous results and time delays.

13. Laboratories should perform DSTs or refer isolates to reference laboratories for DSTs in the following circumstances:

    - 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.[14,15]

The minimum DSTs that should be performed are for isoniazid (high- and low-level concentrations as appropriate), rifampicin, ethambutol, +/– streptomycin.

14. All positive culture and DST results that will affect patient management should be phoned and faxed to the treating doctor and the responsible TB control unit as soon as the results are available. For example, the initial results on all new patients, relapses and failure cases must be phoned and faxed directly to the treating doctor. Repeat results on subsequent specimens from the same episode can be sent in printed form.

15. Laboratories should aim to report positive MTBC cultures within an average of 14–21 days from time of specimen reception. These TATs are achievable using modern broth-based culture systems.

16. All MTBC isolates should be retained for at least six months by the referring laboratory and for at least three years by the reference laboratory.

17. Reference laboratories should also provide directly or through collaborative agreements, access to molecular epidemiological tools (e.g. restriction fragment length polymorphism – RFLP, spoligotyping, variable number tandem repeat – VNTR) so that outbreak strains and laboratory cross-contamination episodes can be recognised.

18. Microbiological laboratories performing TB cultures should ensure that they, or the reference laboratory to which their cultures are referred, include all positive culture results in the national figures collated through the MRL network.

19. Laboratories performing TB cultures must participate in a recognised QAP program. The RCPA QAP program distributes 8–10 specimens for mycobacterial culture per year. A review of laboratory procedures should be instituted if more than one false-positive or false-negative QAP culture result occurs per year.

20. Laboratories performing TB cultures should liaise closely with their state MRL. This liaison may be demonstrated by consultation over positive cultures, attendance at clinical meetings, and/or staff visits to the MRL. Such liaison is particularly important if the laboratory does not have the minimum recommended workload or is not fulfilling QAP or other requirements.

21. The ASM Special Interest Groups for Media Quality Control and Mycobacteriology are developing guidelines for assuring the quality of solid media used in mycobacteriology laboratories.[16] Laboratories must comply with this document particularly when the final version is published.

Referral of non-tuberculous mycobacteria cultures

With the low incidence of TB in Australia, the culture, identification and susceptibility testing of non-tuberculous mycobacteria represents an increasing proportion of the workload for the MRL network. These investigations should not be performed on every NTM isolate (as many represent colonisation or contamination) but only when clinically relevant. Diagnostic criteria[17] have been described for determining the significance of a pulmonary NTM isolate, particularly *M. avium* complex (MAC) and *M. abscessus*. These criteria should be applied when deciding which NTM to refer for identification:

1. If three sputum/bronchial washings are available in the previous 12 months: three smear-negative culture-positive results, or two culture-positive results of which one is also smear-positive;
2. If only one bronchial wash is available, the smear and/or culture show a heavy burden (2+–4+) of NTM;
3. If the above investigations are non-diagnostic, a lung biopsy yields a NTM or shows granulomatous inflammation and/or AFB.

Mycobacterial culture laboratories must therefore consider these diagnostic criteria and liaise with the requesting clinician before forwarding a NTM isolate to an MRL for further identification.

Susceptibility testing of NTM is a controversial issue. There are no data to show that DST results predict clinical outcome for many NTM infections. Furthermore, NCCLS has only recently released recommendations to standardise the performance of NTM DST.[14,15] Hence, mycobacterial culture laboratories should only expect an MRL to provide DST results in the following circumstances:[14,15]

Clarithromycin susceptibility testing for MAC

1. Clinically significant isolate from a patient who has received previous macrolide therapy (i.e. clarithromycin or azithromycin);
2. patients who have developed MAC bacteraemia on macrolide preventative therapy;
3. patients failing or relapsing on macrolide therapy; and

4. baseline isolates from significant MAC infections may also be tested (or stored and tested retrospectively if the patient does not respond to treatment).

Mycobacterium kansasii

1. All initial isolates of *M. kansasii* should be tested against rifampicin;
2. for patients failing or relapsing on treatment; and
3. for rifampicin-resistant isolates, the following antibiotics should be tested: isoniazid, ethambutol, rifabutin, clarithromycin, ciprofloxacin, streptomycin, and co-trimoxazole.

Rapidly growing non-tuberculous mycobacteria

All clinically significant rapid growers should be subjected to testing against: amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, imipenem, and a sulphonamide. Tobramycin should also be tested for *M. chelonae* isolates only.

Susceptibility testing in other circumstances may be performed following close communication between the treating clinician, the mycobacterial culture laboratory, and the MRL, and with reference to the published guidelines on NTM DST.[14,15]

Guidelines for laboratories performing susceptibility tests

Laboratories performing mycobacterial drug susceptibility testing must meet the requirements (i.e. facilities, equipment and work practices) for laboratories performing mycobacterial cultures. Laboratories performing susceptibility tests should have PC3 facilities or have building plans to acquire PC3 facilities by 2007.

Drug susceptibility testing for *Mycobacterium tuberculosis*

The DSTs must be performed using a broth-based 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.[2,3] The DSTs themselves can generally be completed within 7–14 days of obtaining the initial *M. tuberculosis* isolate from the primary cultures.

Drug susceptibility tests must be performed in the following circumstances:

- 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.[14,15]

The minimum DSTs that should be performed are for isoniazid (high- and low-level concentrations as appropriate), rifampicin, ethambutol, +/– streptomycin. The critical concentrations to be employed for these antibiotics in the BACTEC radiometric method are listed in Table 1. Revised guidelines on breakpoint concentrations may be required when the BACTEC system is superseded by non-radiometric methods (e.g. the MGIT 960 has received FDA approval for TB DST).

Supplemental tests to determine low-level resistance should be performed for isoniazid and may also be performed for ethambutol (Table 1). For isolates demonstrating isoniazid resistance at the critical concentration but susceptible at the higher concentration, USA authorities 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'.[14,15] Australian laboratories could consider adding a similar comment in these circumstances after discussion with their TB Chest Clinic specialists.

Table 1. Critical concentrations for first- and second-line drug susceptibility testing of *Mycobacterium tuberculosis* using the radiometric BACTEC technique

Drug	Critical concentration (µg/ml)	Supplemental tests (µg/ml)
Isoniazid	0.1	0.4
Rifampicin	2.0	
Ethambutol	2.5	7.5
Streptomycin	2.0	6.0
Capreomycin	1.25	
Ethionamide	1.25	
Kanamycin	5.0	
Amikacin	1.0	
Clofazimine	0.5	
Ofloxacin	2.0	
Rifabutin	0.5	

Second-line drug susceptibility tests should be performed on:

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

The critical concentrations to be employed for second-line agents in the BACTEC radiometric method are also listed in Table 1. These breakpoints were determined in a multi-centre validation of second-line drug susceptibility testing by the radiometric BACTEC 460 technique.[18] No critical concentration could be recommended for cycloserine because of inconsistent results. The clinical significance of rifabutin 'susceptibility' in the setting of rifampicin resistance remains uncertain. Some clinicians argue that rifampicin resistance implies clinical resistance to all rifamycins and that rifabutin-susceptible rifampicin-resistant isolates merely reflect the use of an incorrect breakpoint for rifabutin.

Pyrazinamide susceptibility testing remains controversial and difficult. The majority of Australian reference laboratories use Wayne's pyrazinamidase (PZase) method to infer pyrazinamide susceptibility or resistance. This method is technically demanding particularly with drug-resistant strains and can give false-susceptible results if resistance is conferred by a mechanism other than PZase mutation. Only one Australian laboratory uses the expensive BACTEC pyrazinamide vials. USA recommendations suggest that, if pyrazinamide resistance rates are low, pyrazinamide susceptibility testing need only be performed as a second-line test on multi- or poly-resistant strains.[14,15] In 2000, only 8 (1.0%) of 768 *M. tuberculosis* isolates in Australia were reported as pyrazinamide resistant. Formal pyrazinamide testing in Australia could therefore be considered a second-line test. The informal PZase surveillance performed by MRLs should provide continued justification for this recommendation.

Drug susceptibility testing for other slow-growing mycobacteria

Clarithromycin and azithromycin are the only drugs where a correlation has been demonstrated between *in vitro* DST results and clinical outcome for MAC disease. Hence, MAC DSTs should only be performed for this drug class. The BACTEC radiometric system provides accurate and reliable results for MAC DST. Laboratories are referred to the NCCLS recommendations and manufacturer's instructions for performing these tests.[14,15]

The NCCLS recommendations also provides guidelines for performing DSTs for *M. kansasii* using the radiometric BACTEC system.[14,15] In general, susceptibility testing for other slow-growing mycobacteria have not been properly standardised or validated, and should only be performed in rare instances with close consultation between the treating physician and the laboratory.

Drug susceptibility testing for rapid-growing mycobacteria

Drug susceptibility testing for rapid-growing mycobacteria (RGM) can be performed by: broth microdilution, E-test, agar disc elution, and agar disc diffusion. The NCCLS recommends the broth microdilution based on a multi-centre study that evaluated the inter-laboratory reproducibility of broth microdilution for commonly encountered pathogenic RGM.[15,19] However, broth microdilution remains problematic: requiring expertise with the recommended method, requiring knowledge of the expected susceptibility patterns of different RGM, and difficulties with trailing endpoints.[19]

Agar disk diffusion is based on the Kirby-Bauer technique using commercially available antibiotic disks. Its major advantages are cost and ease of use, plus the ability to view colonial morphology. Unfortunately, the disadvantages are substantial, particularly with the interpretation of partial zones of inhibition when the disk concentration is close to the MIC breakpoint. Furthermore, many of the newer drugs (e.g. fluoroquinolones, clarithromycin, imipenem) have not had disk susceptibility validated against an MIC method.[19]

Although broth microdilution is recommended by the NCCLS for the susceptibility testing of RGM, the inherent technical and interpretive difficulties have discouraged the widespread uptake of this method by reference laboratories around the world. The AMRLN laboratories continue to use the agar disc diffusion method complemented by identification to species level. The AMRLN laboratories will continue to review these alternative DST methods for RGM (including E-test) and will adopt the preferred practical methodology when an international consensus is reached.

Guidelines for nucleic acid amplification tests

General requirements for a microbiology nucleic acid amplification facility

The National Pathology Accreditation Advisory Council has published standards and guidelines for laboratories performing NAAT.[20] The NPAAC document addresses specimen collection, transportation, reagent preparation, nucleic acid extraction, amplifi-

cation, 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. The efficiency and quality of DNA extraction impacts greatly on the final test result. The extraction methods performed on various specimen types must be documented in the laboratory manuals and validated.

3. All NAAT methods must be properly validated before routine use. When a commercial test is used according to the manufacturer's instructions, no re-validation is required. Modified commercial tests and 'in house' methodologies must be validated as outlined in the NPAAC publication *Requirements for the validation of in-house* in vitro *diagnostic devices (IVDs).*

4. 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.

External quality assurance programs in the USA have demonstrated that laboratories performing TB NAAT but not conforming to these basic requirements have higher rates of false-positive reactions despite using FDA-approved commercial assays.[21]

Special considerations for a tuberculosis nucleic acid amplification facility

Brief literature review

Nucleic acid amplification tests for *M. tuberculosis* have the potential to rapidly determine whether a patient has TB, whether TB treatment is necessary, and whether infection control and contact tracing investigations are required. The features of some commercial NAAT are summarised in Table 2. Though theoretically able to detect a single copy of TB DNA with high specificity, NAAT has proven to have variable sensitivity compared with culture, particularly when investigating smear-negative respiratory specimens (Table 3). The predictive values of NAAT and the pre-test probability of TB in the patient population must therefore be considered when order-

Table 2. Description of some commercial nucleic acid amplification tests.*

NAAT	Method	Target	Sample volume (µl)	Detection	Assay time (hours)	Automation	IAC	FDA approval
AMTD2	TMA	16S RNA	450	Chemiluminescence	2.5	No	No	Yes
AMPLICOR	PCR	16S DNA	100	Colorimetric	6	Yes	Yes	Yes
DTB	SDA	IS6110	500	Fluorimetric	3	Yes	Yes	No

* AMTD2, Amplified *M. tuberculosis* Direct assay, Gen-Probe Inc, San Diego, Calif.; AMPLICOR *M. tuberculosis* assay, Roche Molecular System, Branchburg, NJ; DTB, BD ProbeTec energy transfer (ET) system, Becton Dickinson Biosciences Microbiology Products, Sparks, Md; TMA, transcription mediated amplification; SDA, strand displacement amplification; IAC, internal amplification control. Modified from Relevance of commercial amplification methods for direct detection of *Mycobacterium tuberculosis* complex in clinical samples.22

Table 3. Performance characteristics of some commercial nucleic acid amplification tests*

NAAT	Sensitivity (smear-positive, %)	Sensitivity (smear-negative, %)	Specificity (%)
AMTD2	90–100	63.6–100	92.1–100
AMPLICOR	87.5–100	17.2–71.7	91.3–100
DTB	98.5–100	33.3–100	96–99.8

* Modified from Relevance of commercial amplification methods for direct detection of *Mycobacterium tuberculosis* complex in clinical samples.22

ing and interpreting NAAT tests. Furthermore, the performance of NAAT on non-respiratory specimens has not been properly measured or validated.

Recognising the performance limitations of TB NAAT, the American Thoracic Society conducted a workshop to determine the appropriate use of these tests based on various clinical, laboratory and public health considerations.[23] These issues include:

- the expense of performing NAAT;
- the laboratory preparedness to perform NAAT on a regular basis;
- the cost-benefit of NAAT testing; and
- the interpretation of discrepant smear and NAAT results, particularly AFB-positive NAAT-negative results (i.e. such results may more likely represent the presence of NTM or a false-negative TB result depending on the patient's pre-test probability).

A model was constructed determining when the result of the NAAT test would produce a clinical or public health action. This model relied upon the pre-test clinical suspicion of TB, the AFB microscopy result, and the NAAT result. Treatment, isolation and contact tracing decisions were largely unaffected by NAAT results in smear-positive patients considered at high risk of TB and in smear-negative patients at low-risk of TB. Hence, NAAT testing in these circumstances may represent an inappropriate use of healthcare resources. In contrast, NAAT testing did affect clinical and public health decision-making in smear-negative patients at risk of TB and in smear-positive patients considered at low risk.

The Centers for Disease Control and Prevention updated their guidelines for TB NAAT testing and result interpretation.[24] These guidelines stated that the appropriate number of specimens to test with NAAT will vary depending on the clinical situation, the prevalence of TB, the prevalence of NTM, and laboratory proficiency. An algorithm was presented suggesting which specimens to test, when to perform tests for PCR inhibition, and the interpretation of discrepant smear- and NAAT results.

Basic principles about NAAT testing

The above preamble demonstrates that NAAT testing is only indicated in particular circumstances (e.g. smear-negative patients considered at high risk of TB), that these circumstances are the exact instances where NAAT performance is imperfect (e.g. NAAT detects only one-half to two-thirds of smear-negative TB patients), and that further clinical, public health, and economic research is required to determine the proper indications for TB NAAT testing. In the meantime, clinicians and laboratory staff must recognise the following principles:

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 (e.g. cerebrospinal fluid) should not be preserved for NAAT if this compromises the ability to perform established tests of better diagnostic utility (e.g. culture).[24]
5. Experience is limited with NAAT on non-respiratory specimens and such testing has not been approved by the FDA.[23,24] Again, close clinical and laboratory consultation is required before deciding to perform NAAT on a non-respiratory specimen and particularly when interpreting the result.

A proposed 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, and the laboratory size and resources.

One Australian MRL has developed the following guidelines that others could consider and modify depending on their local circumstances.

The use of NAAT for screening specimens from patients with suspected TB should be limited to:

- respiratory smear-positive specimens where the result is likely to influence clinical (treatment) and/or public health (isolation, contact investigation) decisions;
- respiratory smear-negative specimens from a patient with a high probability of TB, when prompt management and public health decisions are required; and
- selected non-respiratory specimens (e.g. meningeal, some tissue biopsies) where a prompt management decision is necessary (recognised that such tests have not been validated or approved).

The use of NAAT is considered inappropriate in the following instances:

- when a patient is respiratory smear-negative and has a low probability of TB;
- when a patient is respiratory smear-positive and has a very high probability of TB; and
- paucibacillary non-respiratory specimens (e.g. pleural fluid, ascitic fluid).

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 *Safety in laboratories – Microbiological aspects and containment facilities* describes the detailed responsibilities of each of these groups.[8]

Personnel working in mycobacteriology laboratories require:

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

All new staff should have a two-step tuberculin skin test (TST) performed. An initial positive 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 (e.g. 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 (i.e. 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 find an alternative work environment within the microbiology laboratory.

Finally, the Australian/New Zealand Standard 2243.3 recommends vaccination as an additional safety measure for personnel working with *M. tuberculosis.*[8] The efficacy of BCG remains controversial with reported protection levels varying between 0–80 per cent.[25,26] 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 (e.g. those working in laboratories performing DSTs) should be offered BCG after counselling about the advantages and disadvantages of the vaccination.

Acknowledgments

The original draft was developed in consultation with Richard Lumb, Jan Lanser and other staff at the Institute of Medical and Veterinary Science, Adelaide. The guidelines were refined by expert scientists from the various MRLs (i.e. David Dawson, Chris Gilpin, Frank Haverkort, Peter Howard, and Aina Sievers). Valuable input was also obtained from members of the ASM Mycobacterium SIG, Standards Australia, PHLN, RCPA, and other interested parties, through a consultative process.

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