4. Laboratory Diagnosis of Tuberculosis

4.1 Role and Goals of the Modern TB Laboratory

The microbiology laboratory makes a key contribution to TB control in terms of diagnosis, infection prevention and control and management of the disease. The detection and isolation of mycobacteria, identification of the mycobacterial species or complex isolated and the determination of susceptibilities of the organism to anti-mycobacterial drugs are key functions of a modern TB laboratory.[110] The gold standard for diagnosing active disease is by microbiological identification of TB by culture. Molecular typing of isolates contributes significantly to understanding the epidemiology of TB. Population based molecular epidemiology studies have been used to evaluate TB control efforts, providing insights into transmission dynamics, enhancing contact tracing and aiding the detection of laboratory cross-contamination.[111] The role and importance of skilled and experienced staff cannot be over emphasised.[112]

In 1993, WHO declared TB a global emergency in response to an increase in cases after nearly a century of decline. Targets for achieving improved control were developed which involved diagnosing a minimum of 70% of individuals with sputum smear positive TB and curing at least 85%. In Ireland, important legislative changes have occurred since the publication of the 1996 TB guidelines.[113] In 2003, the Infectious Diseases Regulations 1981 were amended by the Infectious Disease (Amendment) (No. 3) Regulations 2003 (S.I. No. 707 of 2003), stating that a clinical director of a diagnostic laboratory shall have regard to the case definitions for notification of infectious diseases.[21] The DPH or designated medical officer should be notified of the identification of a TB case ideally within one working day.

Recommendation:

It is a mandatory requirement for clinical directors of diagnostic laboratories to notify cases of active TB disease to the medical officer of health (director of public health or designate).

Culture is necessary to achieve the "gold standard" for the diagnosis of active tuberculosis disease.

Levels of service

Before offering mycobacteriology services, each laboratory should assess the capacity and capability of the level of services performed. Clinical laboratories offering a mycobacteriology service are divided into three major categories of service (table 4.1). This follows recommendations by CDC and the American Thoracic Society (ATS) stating that laboratories interpreting acid fast stained smears should process at least 10-15 specimens per week to maintain proficiency and those processing specimens for culture should handle a minimum of approximately 20 specimens per week.[114-117] The majority of laboratories in Ireland are Level 2.

Table 4.1: Levels of service for diagnostic microbiology laboratories

Laboratory service level	
Level 1	Collection and transport of specimens; preparation and examination of smears for AFB.
Level 2	Procedures of level 1, plus procedures for the isolation of *Mycobacterium* species. Identification of *M. tuberculosis* complex may or may not be performed.
Level 3	All procedures in level 2, plus identification of all species of mycobacteria. The determination of drug susceptibility for all *Mycobacterium* species and typing of *M. tuberculosis* complex should be performed at level 3.

Recommendation:

Microscopy and culture for TB should only be performed in those laboratories where there is sufficient throughput to ensure proficiency.

In 1993, Tenover,[118] Huebner[119] and subsequently CDC[120] and Styrt[121] recommended by way of "goals" (not mandates or regulations) that a TB laboratory should report within certain time frames as shown in table 4.2.

Table 4.2: TB laboratory service goals

Goals	Time frame*
Report results of acid fast stains	Within 24 hours
Detect growth of mycobacteria in liquid medium	Within 10 days
Identify *M. tuberculosis* complex by mycolic acid pattern, AccuProbe or Bactec NAP test	Within 3 weeks
Determine and report susceptibilities of new *M. tuberculosis* isolates to primary drugs	Within 3-4 weeks

*Within receipt of specimen in the laboratory

Recommendation:

Laboratories should aim to meet the "goals" set down by CDC and others.

The Irish Mycobacteria Reference Laboratory (IMRL)

The IMRL was established in 2001 to provide a timely reference service in relation to the diagnosis and treatment of TB (see appendix 7 for contact details). Advice is available from a consultant microbiologist, a respiratory physician with expertise in the treatment of TB and senior laboratory scientists. The Department of Health and Children has set out the core functions that the reference laboratory service will be required to perform once it is fully developed. The IMRL functions include:

- Identification of mycobacterial isolates
- Sensitivity testing of isolates to anti-mycobacterial drugs
- Assistance with isolation of mycobacteria in difficult cases
- Provision of advice to clinicians and laboratories
- Provision of clinical advice on diagnosis, treatment and infection prevention and control
- Provision of a molecular diagnostic service for rapid identification
- Molecular typing of *M. tuberculosis*
- Training of medical/technical staff
- Research
- Provision of epidemiological information as required, subject to agreement with the HPSC.

Some of the above services are currently not available. As the IMRL is developed, all of the services outlined above will be provided.

Recommendation:

All mycobacterial isolates should be referred to the Irish Mycobacteria Reference Laboratory (IMRL) for identification and susceptibility testing once its new facility is opened.

Recommendation:

All *M. tuberculosis* complex isolates should be referred with immediate effect to the IMRL for molecular typing where typing is now offered.

Recommendation:

The results of all *M. tuberculosis* complex isolates which have already had identification, susceptibility and molecular typing performed should be forwarded to the IMRL for incorporation into a national repository of *M. tuberculosis* complex isolates.

4.2 Specimens

All specimens should be collected and submitted in sterile, clean, plastic, leak-proof, disposable, wide-mouthed, appropriately labelled, laboratory approved containers, without any fixative. Generally, transport media or preservatives are not needed owing to the robust nature of mycobacteria.[122] Swabs are not optimal for the recovery of AFB.[122] Ideally specimens should be procured before chemotherapy is initiated. Even a few days of therapy can obscure the diagnosis because of the failure to recover mycobacteria.[123] Specimens should be transported to the laboratory as soon as possible and refrigerated until processed. The transportation of infectious substances is subject to international legislation, to which laboratories are required to comply (section 4.7).

Rejection of unsuitable specimens

The best mycobacteriological laboratory practices are frustrated if a poor or unsuitable specimen is presented for examination. Processing of inappropriate clinical specimens for mycobacteria can be wasteful, may be rejected and the clinician notified.

Examples of inappropriate specimens are as follows:

- Insufficient amount submitted
- Specimens consisting of saliva
- Dried swabs
- Pooled sputa or urine
- Broken sample containers
- Interval too long between specimen collection and processing[124]
- Analysis of urine specimens for the diagnosis of pulmonary TB.

Recommendation:

Reasonable efforts should be made to obtain the best quality sample possible depending on site of disease and to deliver it in a timely fashion to the analysing laboratory.

Sputum specimens

A direct relationship between mucopurulent sputum and positive results has been demonstrated.[125] Therefore, patients should be instructed as to the proper method of sputum collection. Ideally a 5-10ml specimen[122;126] collected early in the morning[§§] on three consecutive days (with a minimum of one early morning specimen) prior to the commencement of treatment, if possible, is required (or failing that, within seven days of commencement of treatment).[123;126;127] Failure to isolate *M. tuberculosis* complex (MTC) from appropriately collected specimens from persons suspected of having a pulmonary diagnosis does not exclude a diagnosis of active TB. Depending on the clinical features and differential diagnosis, other diagnostic testing such as induced sputa, bronchoscopy lavage and biopsy should be considered before making a presumptive diagnosis of culture negative TB.[128]

Bacteriological monitoring

Bacteriological monitoring after diagnosis may be required to assist with a decision to discontinue isolation. The period of time a patient on effective therapy takes to become non-infectious varies. Patients who have unrecognised or inadequately treated drug-resistant TB may remain infectious for weeks or months.[129] Laboratories can be overwhelmed with specimens obtained for the purposes of demonstrating smear conversion in smear positive patients. This can also contribute to an increase in false positive cases in the laboratory. It should be noted that beyond 12 weeks of treatment, 63% to 73% of patients with persistently positive smear results have negative culture results. In the absence of MDR-TB and XDR-TB, the persistence of AFB in smears at the end of therapy is not necessarily a treatment failure.[130;131] Laboratories should liaise with clinicians to develop a protocol for processing such specimens. Follow-up sputum specimens for smear and culture should be obtained monthly in patients with drug-susceptible pulmonary disease. For patients with isoniazid- and rifampicin-susceptible TB there is no need to examine sputum monthly once culture conversion is documented (i.e. two negative cultures taken at least 2 to 4 weeks apart).[77] It is recommended that identification and sensitivities are repeated in cases who are still culture positive at ≥ two months. If the patient has isoniazid and/or rifampicin resistant TB, sputum cultures should be examined monthly until the end of treatment.[77] Bacteriological monitoring i.e. culture at the end of treatment is strictly recommended in confirmed cases of TB to assess precisely that the patient has been cured. Culture positive *Mycobacterium tuberculosis* complex (MTC) from each different site should undergo identification and sensitivity testing.

Induced sputum and fibreoptic bronchoscopy

Hypertonic saline can be nebulised for induction of sputum. Bronchoscopy is the next best choice because this procedure provides additional material for study, washings, brushings and biopsy specimens. Induced sputum has been shown to be as sensitive[132] or more sensitive[133;134] than bronchoscopy and is also cheaper to perform. Sputum induction in children has been shown to be safe and useful for microbiological confirmation of TB in children and preferable to gastric lavage.[135] Where facilities permit it may be preferable to gastric lavage.[135]

The bronchoscopy procedure may cause the patient to continue producing sputum for several days. The use of bronchoscopy in evaluating children with TB is not routine but it can provide useful information in selected cases.[136] (See section 8.1. Chapter 8 for contact tracing) (See section 6.5. Chapter 6 for infection prevention and control). These samples should also be collected and examined. [110]

Gastric washings

Gastric lavage may be necessary for those patients, particularly children, who cannot produce sputum even with aerosol inhalation. Fasting early morning specimens are recommended to obtain sputum swallowed during sleep.[124] These specimens should be processed within four hours or neutralised with sodium carbonate or another buffered salt to a pH of 7.0. It has been suggested that for these reasons gastric aspiration is best performed by experienced staff.[137] AFB smear testing is unreliable due to the high prevalence of atypical mycobacteria in gastric lavage. Culture should only be used for further clinical management.

Urine

Urine is an inappropriate specimen for the diagnosis of pulmonary TB. The diagnosis of renal TB, disseminated TB or TB in severely immunocompromised persons can be aided by the culture of 3 early

§§ Early morning sputum: sputum from the first productive cough in the day (after waking)

morning mid-stream urines each with a volume of 40-50ml. Smears of urine are usually negative and may not be cost effective[110] or they may give unreliable results due to the presence of environmental mycobacteria that may be found in the lower urethra.[127] However, if examination of urine is restricted to those patients who are severely immunocompromised, suspected of having renal or disseminated TB, or when haematuria or sterile pyuria has been demonstrated, the value of performing smears may be improved.

Faeces
Faecal specimens are largely used in the detection of *Mycobacterium avium* complex (MAC) from the intestinal tract of patients with HIV/AIDS, in conjunction with specimens from other sites. Interpretation may also be difficult due to the presence of saprophytic AFB frequently present in faeces samples.[127]

Cerebrospinal fluid
Cerebrospinal fluid (CSF) should be examined for protein, glucose and white cell count and differential. Lymphocytosis together with a low glucose and high protein are typical of TB meningitis. The volume of CSF is critical and a minimum of 5ml[110] but up to 10ml is ideally required[126;138] provided it is medically safe to obtain this volume. Studies should be undertaken prior to the administration of anti-tuberculous chemotherapy. There is a low yield of approximately 40% and if feasible it may be worthwhile to perform repeat cultures.

Blood culture
Infection with *Mycobacterium* species became increasingly common as the incidence of HIV infection and AIDS increased. Up to 63% of HIV/AIDS patients with active TB disease have positive blood cultures.[139] Blood culture should be the first step in the routine evaluation of HIV positive patients with suspected TB.[140]

Tissue
When non-invasive procedures have failed to provide a diagnosis, invasive procedures to obtain specimens from lung, pericardium, lymph nodes, bones and joints, bowel, etc. should be considered.[110] **Specimens submitted in formalin are not suitable for microbiological smear and culture.** In patients with haematogenous or disseminated disease, bone marrow biopsy, lung biopsy and liver biopsy for histological examination and culture can be useful.[110] Tissue is preferable to necrotic material or pus, as the latter contain free fatty acids that are toxic to mycobacteria.[127] A caseous portion should be selected if possible as the majority of organisms will be found in the periphery of a caseous lesion.[126] Pleural biopsy shows granulomatous inflammation in approximately 60% of patients.[110] Minute amounts of biopsy material may be immersed in a small amount of sterile saline to stop them drying out.

Body fluids
Pleural, peritoneal, synovial and pericardial fluids should be aseptically collected by aspiration or during surgical procedures and transported to the microbiology laboratory immediately. It should be noted that detection of AFB in smears of culture positive pleural fluids is extremely low at between 0 to 1%[141] and the sensitivity of nucleic acid amplification tests (NAAT) has been shown to be in the order of only 27 to 32% using fully commercially available methods (see section on nucleic acid amplification tests).[142]

4.3 Specimen Processing

Microscopy
Acid-fast microscopy is "the microscopic examination of stained smears for the presence of organisms that retain the primary stain when the smear is decolourised with an acid alcohol solution". Fluorescence staining,[143] of clinical material using auramine-o or auramine-rhodamine is to be preferred over Ziehl-Neilson (ZN) staining because it is quicker and easier to read,[124;144;145] whilst the ZN is more appropriate in determining microscopic morphology of bacilli in positive TB cultures. Factors affecting the sensitivity of smears are many and include the staining technique, centrifugal force, decontamination technique used, the infecting species, reader experience and the prevalence of disease in the population being tested.[110;145;146]

Key points relating to microscopy:

- Results should be available within one working day
- Microscopy of clinical material (by either Auramine or Ziehl-Neilson (ZN)) is the easiest, most cost-effective and rapid procedure for detecting mycobacteria

- Both viable and non-viable organisms will stain acid fast
- Microscopy has a sensitivity of approximately 90% for the most infectious cases of presumed TB. It therefore provides information to support respiratory isolation of patients and other infection control measures that prevent transmission of disease.
- The grading of a smear can give some indication to the probability of a positive culture[124;147] (see table 4.3)
- The minimum number of bacilli necessary to produce a result has been estimated to be between 5,000 and 10,000 per millimetre of sputum. The incremental yield of AFB from serial smear examination has been shown to be 80-82% for the first, 10-14% for the second and 5-8% for the third specimen.[124;147]
- 50-80% of patients with pulmonary TB will have positive smears[110]
- It is a useful tool for initiating treatment and monitoring the progress of anti-TB drug therapy
- Fluorescence microscopy is more sensitive and as specific as conventional AFB microscopy[148]
- A minimum of 75 fields (250x) or 300 fields (1000x) should be examined by fluorescence and ZN respectively[145]
- Re-examination of negative smears from specimens with positive culture can dramatically decrease a laboratory's rate of false negative smears[144]
- Supervisory review of doubtful results in newly diagnosed patients can markedly decrease the incidence of false positive smears[144;145]
- Optimal results for sputum are obtained when auramine phenol stain is applied to a liquefied, concentrated sample and examined before the decontamination process[149]
- In laboratories with good expertise the predictive value of a positive smear can be as high as 90% and the predictive value of a negative smear 96% (based on good quality specimens)[144]
- The laboratory should participate in an external quality assurance scheme e.g. UK NEQAS AFB scheme.

Table 4.3: Relationship of acid-fast smear and culture yield

	No. of AFB seen by staining method and magnification				
Report	***ZN stain***	***Fluorochrome***		***Estimated concentration of bacilli***	***Probability of a culture positive result***
	X 1,000	X 250	X 450		
Doubtful; repeat	1-2/300F	1-2/30F	1-2/70F	5,000-10,000	50%
1+	1-9/100F	1-9/10F	2-18/50F	About 30,000	80%
2+	1-9/10F	1-9/F	4-36/10F	About 50,000	90%
3+	1-9/F	10-90/F	4-36/F	About 100,000	96.2%
4+	> 9/F	>90/F	>36/F	About 500,000	99.95%

Decontamination

Many of the specimens sent for the isolation of *Mycobacterium tuberculosis* are contaminated with commensal flora originating from the specimen site. This is particularly true of specimens from the respiratory and genitourinary tracts. Sodium hydroxide is the most common decontaminating agent used in mycobacteriology. A contamination rate of between 2% to 5% is deemed acceptable.[115] An incidence of less than 2% may suggest excessive decontamination and above 5% may suggest that cultures which might contain mycobacteria may be overgrown by commensal organisms.[110]

Samples from cystic fibrosis (CF) patients present a significant problem in that *Pseudomonas aeruginosa*, isolated from approximately 80% of CF patients can hinder and even prevent the recovery of

Mycobacterium tuberculosis.[150] A two-step approach is advised where samples are initially decontaminated with N-acetyl-L-cysteine NaOH (NALC/NaOH) and only those samples which remain contaminated are subjected to a second round of decontamination with NALC/NaOH and oxalic acid. Results suggest that this protocol could improve the recovery of mycobacteria from heavily contaminated specimens.[151]

Culture

Culture is the bedrock and most reliable tool for the diagnosis of TB[152] and has a detection limit of 10^1 to 10^2 viable organisms.[124] It is essential so that isolates are available for susceptibility testing, the results of which are necessary for the proper management of the patient. A confirmed case of TB is a case with culture confirmed disease due to *M. tuberculosis* complex.[23]

Isolates are also necessary in genotyping studies which are required for epidemiological investigations and tracking laboratory cross-contamination.[153] *Mycobacterium tuberculosis* is a slow growing bacterium and special media and procedures, not required for the culture of other organisms, are necessary.[127] Agar based and egg based media, Middlebrook broths or solid media, the "traditional" methods of culture, have been superseded by the semi automated Bactec 460 and more recently by the continuous automated monitoring in liquid culture techniques (CAMLiC)[154] e.g. the Bactec MGIT960™, MB/BacT Alert 3D-™ and the VersaTREK™ systems. These are now the basis of the "gold standard" in the isolation, culture and definitive diagnosis of mycobacterial disease.

Each of the Bactec 460TB and CAMLiC systems has advantages and disadvantages and the choice of system is often based on non-microbiological factors, e.g. size of equipment, quality of manufacturer's service and maintenance or in-built electronic data management systems. The microbiological factors include:

- Sensitivity of the detection system
- Performance in recovery of *Mycobacterium tuberculosis* complex (MTC) and non-tuberculous mycobacteria (NTM)
- Speed to positivity
- Ability to perform susceptibility tests to anti-mycobacterial drugs and
- Chemical agents, safety and cross-contamination.

It is emphasised that in order to get optimal recovery of mycobacteria from clinical specimens using a CAMLiC system, a combination of liquid and solid media is essential especially in non-specialised laboratories with a low incidence of *M. tuberculosis*.[155-157]

Recommendation:

It is recommended that solid media be used in combination with a liquid culture system.

Nucleic acid amplification tests (NAAT)

CDC has produced an updated algorithm which offers useful guidance on the use of nucleic acid amplification tests (NAAT)[158] directly on specimens. CDC now recommends that NAAT 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 or TB control activities such as contact tracing.[158]

Recommendation:

All those wishing to undertake NAAT on suspected cases of pulmonary TB should seek advice from the local consultant microbiologist. It is recommended that NAAT should be made available at the IMRL.

Clinicians need to be aware of the limitations of these tests particularly in relation to the types of specimens analysed, the commercial system in use and the quality assurance programmes in place.[159-162] Application of NAAT for the diagnosis of TB in sputa, CSF, and the detection of rifampicin and isoniazid resistance in primary and reference specimens should be provided only where there is an adequate infrastructure, primarily sufficient space for a unidirectional workflow and dedicated equipment.[163] Standardised methods with adequate and appropriate positive and negative controls should be used.[112]

A cost effective approach is to base these tests in laboratories in Ireland with expertise in molecular biology. For procedural and economic reasons, NAAT might be impractical for laboratories with a small volume of testing. Referral of samples for NAAT to high-volume laboratories might be preferable to improve cost-efficiency, proficiency and turnaround times.[158]

4.4 Processing of Positive Cultures

Identification methods

Phenotypic methods

Microscopic and colonial morphology and other growth characteristics are useful in making a preliminary identification of an acid-fast bacillus. Colonies of the tubercle bacilli are described as being "rough, tough and buff" on solid media and colonies tend not to emulsify easily for making smears. MTC form serpentine chords in liquid media and are easily observed with ZN staining. These characteristics can lead to the most relevant tests being performed on each isolate to obtain identification.[127]

Probe techniques

Probe detection methods such as the AccuProbe, targeting ribosomal RNA gene, can identify the MTC, *M. avium*, *M. intracellulare*, *M. avium complex* (MAC), *M. gordonae* and *M. kansasii*. The technique is relatively simple and the results are available within hours. The specificity has been shown to be 100% but the sensitivity can vary within the species or species complexes.[124]

PCR and restriction endonuclease analysis

In 1993, Telenti[164] modified the Polymerase Chain Reaction (PCR)-Restriction Endonuclease Analysis technique, subsequently known as the PRA method, to allow for the rapid identification of mycobacteria to the species level. The method has been used extensively for mycobacterial identification. The principal disadvantage is that it is not commercialised and does not have US Food and Drug Administration (FDA) approval or carry CE.[♦] markings.

PCR and reverse hybridisation techniques

New molecular biology techniques based on PCR and reverse hybridisation procedures have recently been marketed for mycobacterial identification. The hybridisation technique is performed on nitrocellulose strips onto which probe lines are fixed in parallel. This format enables simultaneous detection and identification of different mycobacterial species. At present, two systems with this design are commercially available in Europe, the INNO-LiPA™ Mycobacteria v2 test, designed to amplify the mycobacterial 16S-23S rRNA spacer region, and the HAIN GenoType™ Mycobacteria assay, targeting the mycobacterial 23S rRNA.[165] Both methods can be completed within two working days and allow precise identification of the majority of mycobacteria usually isolated in clinical laboratories. They are expensive to perform but this can be offset by the reduction in turnaround and labour times. These methods are available in the United States as FDA-approved tests and bear the CE mark in the European Community.

DNA sequencing

The availability of DNA sequencing technologies constitutes a great benefit for mycobacterial identification, owing to the slow growth of these organisms. Recent improvements in automation of target amplification and sequence analysis have led to the practical implementation of DNA sequencing in some clinical laboratories.[124] The technique requires expensive equipment and is best reserved for reference laboratories.

[♦] The CE marking (also known as CE mark) is a mandatory conformity mark on many products placed on the single market in the European Economic Area (EEA).

Mycobacterium tuberculosis complex (MTC)
The *Mycobacterium tuberculosis* complex now consists of the following strains; *M. tuberculosis* sensu stricto, *M. bovis*, *M. bovis* BCG, *M. africanum*[166] *M. canettii*[167], *M. bovis* subsp. *caprae*[168;169], *M. microti*[170], and *M. pinnipedii*.[171] All are known to cause infections in humans but they differ in their primary host, geographic range and pathogenicity. *M. microti* grows very slowly, often requiring up to 16 weeks incubation in liquid and solid culture media.

Various methods may be used in order to differentiate members of the MTC. Specific inhibitors can be added to a culture medium and growth or the lack of growth determined e.g. thiophen-2-carboxylic hydrazide (TCH). *M. tuberculosis* is resistant to TCH while the other members of the complex are sensitive. Similarly, susceptibility to pyrazinamide is useful in differentiating *M. bovis* from other members of the complex in the majority of cases.

More recently, a new commercially available DNA strip, GenoType MTBC, has been developed and evaluated.[172;173] Results demonstrated that the assay could unambiguously differentiate all of the MTC, with the exception of *M. tuberculosis*, *M. africanum* type II and *M. canettii*. The latter is considered to be a smooth variant of *M. tuberculosis*[167] and *M. africanum* II has not been successfully differentiated from *M. tuberculosis* using molecular techniques suggesting that it likely represents phenotypically atypical *M. tuberculosis* strains.[174]

The GenoType MTBC was found to:
- Enable a very rapid identification of the MTC
- Fit into the work flow of a routine laboratory and
- Be conducted in laboratories that do not carry out sophisticated biochemical tests for differentiation of the MTC.

The results of these studies challenge the use of biochemical characteristics for classifying these organisms in diagnostic laboratories.[174]

Susceptibility testing of *M. tuberculosis*
CDC has recommended that mycobacteriology laboratories work towards the goal of reporting first-line susceptibility results for MTC within three to four weeks of receipt of the initial diagnostic specimen. Ideally, susceptibility results should be available within seven to 14 days after isolation of an MTC isolate. *M. tuberculosis* complex resistance is defined as "a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come into contact with the drug".[175] Methods of drug susceptibility are not designed merely to detect drug resistant mutants but are also to show that the great majority of bacilli in a culture are as susceptible to a given drug as one or more known susceptible strains. The object of susceptibility testing is therefore to determine whether an isolate is as likely to respond to standard therapy as one or more known susceptible strains.[127]

There are five methods in current use as outlined below:
1. The absolute concentration method which is popular in some parts of Europe
2. The proportional method also used in Europe and using Middlebrook 7H10 agar is the "gold standard" method in the USA
3. The disk diffusion method
4. The resistance ratio method is used in the UK and those countries that are influenced by UK practice and
5. Commercially available systems, including the FDA approved Bactec 460TB, Bactec MGIT 960 and the Versa TREK systems.

The first four methods rely on conventional media and thus have the great disadvantage that there is a long delay before results are available. In developed nations commercially available systems are used, so that results are made available more rapidly.[127] If resistance is detected the test may be repeated for confirmation purposes, however, a report of the initial result should not be delayed while the repeat testing is being performed. The report should indicate that the drug resistance findings are preliminary and confirmatory testing has been initiated.

The first isolate of MTC obtained from every patient should be tested but also each isolate recovered

from each different anatomical site in the same patient. Susceptibility tests should be repeated if there is clinical evidence of failure to respond to therapy or if cultures fail to convert to negative after two months of therapy. For patients with resistant isolates, including resistance to the lower, critical concentration of isoniazid, referral to or consultation with a specialist in TB treatment should be considered.[175]

The application of two commercially available DNA line probe assays, Genotype MTBDR™ and INNO-LiPA Rif™ to detect resistance to isoniazid and rifampicin (HAIN) or rifampicin alone (INNO LiPA) can be performed when there is a strong suspicion of MDR-TB. Neither is 100% sensitive and results must be confirmed by conventional testing.[176] In vitro susceptibility tests are very satisfactory for isoniazid, rifampicin and pyrazinamide and slightly less so for streptomycin. Results from ethambutol (E) and second line drugs may vary depending on the test method used.

Monitoring of anti-mycobacterial drug serum levels
This may be occasionally required in suspected cases of non-compliance, malabsorption or toxicity. This service can be provided by prior arrangement with the Antimicrobial Reference Laboratory in Southmead Hospital, Bristol (see appendix 7 for contact details).

Molecular typing of *M. tuberculosis*
There are currently three prominent methods for typing of *M. tuberculosis* strains. The current "gold standard" is the IS*6110*-based restriction fragment length polymorphism fingerprinting.[177] This technique is technically demanding and requires abundant amounts of growth of isolates. The remaining two methods are polymerase chain reaction (PCR)–based genotyping tests, mycobacterial interspersed repetitive units (MIRU) typing[178] and spoligotyping.[179] In combination, the latter two tests (MIRU typing and spoligotyping) provide a highly discriminatory method to identify strains and will be used in the CDC TB Genotyping Programme to enable rapid genotyping of isolates from every patient in the United States.[180] The IMRL intends to use MIRU typing in the first instance supported by spoligotyping.

Genotyping of isolates can assist in the clinical and public health management of patients in several situations:[112]

- Genotyping allows evaluation of isolates with different patterns of drug susceptibility. The original organism may develop drug resistance during or after anti-TB therapy or the patient may be re-infected with a different strain. The former may be due to non-adherence to therapy or reduced concentrations of anti-TB drugs as a result of malabsorption or drug interaction. The latter may be due to re-infection which would require further contact tracing investigations as a public health issue.
- Evaluation of an outbreak can be more clearly delineated or previously unrecognised contacts detected
- Genotyping can help to establish where resources might best be directed in a TB control programme
- On average 3% of patients from whom *M. tuberculosis* is apparently isolated in clinical laboratories do not have TB. These positive cultures are due to cross-contamination.[181]

4.5 False Positive Cultures

A review of reports of false positive cultures for *M. tuberculosis* showed that false positives were identified in 93% of studies that evaluated more than 100 patients.[181] The median false positive rate was 3.1%, with a range of 2.2% - 10.5%, and even higher rates (13.6%) have since been published.[182] The mechanism of false positive cultures can be many and include contamination of clinical equipment, clerical error and laboratory cross-contamination.

For the purposes of further investigation, the source laboratories should not delay the forwarding of possible false positive *M. tuberculosis* complex isolates to the IMRL. The IMRL should perform DNA fingerprinting on all positive *M. tuberculosis* isolates and not delay the reporting of test results back to the source laboratory. Confirmed false positives should be reported back to the clinicians as soon as possible. Clinicians should balance laboratory test results with their clinical judgement on whether or not a patient has TB and inform the laboratory of any doubts.

Laboratory cross-contamination

Often the most significant laboratory feature is that the false positive culture is the only positive culture from a patient. However, single positive cultures also occur among patients who meet the clinical criteria for a diagnosis of TB. Prospective monitoring of single-positive cultures detected two outbreaks of laboratory cross-contamination that had not been recognised by clinicians or laboratory personnel.[181]

The quality assurance programme for mycobacteriology laboratories should include a plan for the identification and review of possible false positive cultures. Criteria that might prompt a review should include a patient with a single culture positive specimen, cultures with a very low colony count on solid media and isolates with unexpected drug resistance.

Possible causes of laboratory cross contamination

The most common causes of laboratory cross-contamination include:[183]

- Contamination of multiple-use equipment for dispensing reagents
- Aerosols
- Splashing
- Sampling equipment
- Reprocessing of contaminated specimens and
- Mislabelling.

The general principle is to isolate each specimen completely so that there are no opportunities to transfer an inoculum from one sample to another via pipettes, the lips, caps of tubes, splashes or common reservoirs of reagents or containers used for discarded materials. Examples of good laboratory practice include the following:[184]

- Only bring the required numbers of items such as loops, swabs, pipettes, universal containers, etc. into the safety cabinet for each session of work
- When using pipettes to deliver reagents, use a separate pipette for each specimen and each time that the reagent bottle is entered
- Individually wrapped sterile plastic pipettes should be used
- Reagents, such as sodium hydroxide, should be supplied or prepared in individual sterile vials. Use a separate vial to add a reagent to each sample rather than dispensing it from a common container.
- Remove and replace the cap from each specimen tube sequentially during the addition of reagents to specimens, so that only one tube is open at a time and so that tube caps do not become interchanged
- Avoid spills and splashes when decanting supernatants
- Clean and disinfect the exposed surfaces of the safety cabinet after each session of work
- Where possible ensure that specimens are processed in the order of smear negative to smear positive specimens. This requires the keeping of an up-to-date list of known smear positive patients.
- Process positive culture vials only when all the work on specimens has been completed for the day
- At the end of the working day dispose of any used items in the cabinet and clean and disinfect the interior surfaces of the safety cabinet
- If possible, use a separate safety cabinet for specimens and all positive cultures, whether liquid or solid[185]
- Investigate for possible cross-contamination when specimens in proximity to one another become positive. Cross-contamination may not occur with all samples in sequence, such that negative cultures occasionally may be found between positive cultures[183]
- Ensure that written procedures for the processing of cultures include detailed instructions and that staff have a very good understanding of the rationale for all aspects of the procedure.

4.6 Interferon Gamma Release Assays (IGRA)

The TST is a long established test that uses a relatively crude mixture of antigens from *M. tuberculosis* to detect the immune response to infection with *M. tuberculosis* (past or present). As a result false positive reactions can occur because of previous BCG vaccination or sensitisation to non-tuberculous mycobacteria. It has many limitations and requires well-trained personnel to both administer and interpret the test (see chapter 2).

The immune response to infection with *M. tuberculosis* is predominantly a cell mediated immune (CMI) response. As part of this response, T-cells are sensitised to *M. tuberculosis* antigens. Activated effector T-cells produce a cytokine called Interferon gamma (IFN-γ) when stimulated by these antigens. Laboratory blood tests have been developed that detect this release of gamma interferon and are collectively known as interferon gamma release assays (IGRA). The use of selected antigens for the *M. tuberculosis* complex improves specificity by reducing cross-reactivity to the BCG vaccine and to many environmental mycobacteria.[186]

Two separate panels of antigens which simulate the well characterised proteins ESAT-6 and CFP10, are used to optimise the sensitivity of the T-SPOT.TB test, while the QuantiFERON-TB® Gold In-Tube (IT) method additionally incorporates a third antigen, TB7.7 (p4). The T-SPOT.TB and the QuantiFERON-TB Gold® IT are tests for *M. tuberculosis* complex infection (including disease) and are intended for use in conjunction with risk assessment, radiography and other medical and diagnostic evaluations.

T-SPOT.TB (Oxford Immunotec)
T-SPOT.TB is a simplified variant of the enzyme-linked immunospot (ELISPOT) assay technique. The assay is designed for the detection of effector T-cells that secrete the cytokine in response to stimulation by antigens specific for *M. tuberculosis*.[187-190]

Limitations of the TSPOT.TB
According to the manufacturer:

- While ESAT-6 and CFP10 antigens are absent from BCG strains and from most environmental mycobacteria, it is possible that a positive result from the T-SPOT.TB assay may be due to infection with *M. kansasii, M. szulgai* or *M. marinum*. Alternative tests would be required if these infections are suspected
- Variation to the stated pipetting and washing techniques, incubation times and/or temperatures may influence the actual results obtained and should be avoided
- Blood must be collected and progressed into the assay within eight hours
- T-SPOT.TB should be used and interpreted only in the context of the overall clinical picture
- A negative test result does not exclude the possibility of exposure to or infection with *M. tuberculosis*
- Individual users should validate their procedures for collection of PBMCs, enumeration of PBMCs and choice of suitable media to support T-cell functionality during the primary incubation stage of the assay and
- Failure to adhere to the recommended incubation time may lead to an incorrect interpretation of the result.

QuantiFERON-TB Gold®- In-Tube (IT)
QuantiFERON-TB Gold®- In-Tube (IT) (Cellestis, Victoria, Australia) is an in vitro diagnostic test using a peptide cocktail simulating ESAT-6, CFP-10 and TB7.7(p4) proteins to stimulate cells in heparinised whole blood. Detection of IFN-γ by enzyme-linked immunosorbent assay (ELISA) is used to identify in vitro responses to these peptide antigens that are associated with *M. tuberculosis* complex infection.

Limitations of the QuantiFERON-TB Gold® In-Tube (IT)
According to the manufacturer:

- The magnitude of the measured IFN-γ level cannot be correlated to the stage or degree of infection, level of immune responsiveness or likelihood for progression to active disease
- A negative QuantiFERON-TB Gold® IT result does not preclude the possibility of *M. tuberculosis* infection or TB disease: false-negative results can be due to the stage of infection (e.g. specimen obtained prior to the development of cellular immune response), co-morbid conditions which affect immune functions, incorrect handling of the blood collection tubes following venepuncture, incorrect performance of the assay or other immunological variables
- A positive QuantiFERON-TB Gold® IT result should not be the sole or definitive basis for determining infection with *M. tuberculosis*. Incorrect performance of the assay may cause false positive responses. Diagnosing or excluding TB disease and assessing the probability of LTBI, requires a combination of epidemiological, historical, medical and diagnostic findings that should be taken into account when interpreting QuantiFERON-TB Gold® IT results

- A positive QuantiFERON-TB Gold® IT result should be followed by further medical evaluation and diagnostic evaluation for active TB disease (e.g. AFB smear and culture, chest X-ray)
- While ESAT-6, CFP-10 and TB7.7 (p4) are absent from all BCG strains and from most known non-tuberculous mycobacteria, it is possible that a positive QuantiFERON-TB Gold® IT result may be due to infection by *M. kansasii, M. szulgai* or *M. marinum*. If such infections are suspected, alternative tests should be investigated.

Sensitivity and specificity of IGRA

The lack of a gold standard for the diagnosis of LTBI makes it difficult to estimate the sensitivity or specificity of IGRA or TST. Most studies use newly diagnosed active TB as a surrogate for LTBI but there is an inherent problem with this, in that the cell-mediated immune response being measured must have failed, to some extent, in any person with active disease. It has been well documented that in patients with active infection the cell-mediated immune response is often diminished and this possibly explains the finding that all three tests, but particularly TST, has sub-optimal sensitivity.[61;191-193] Of the three, the T-SPOT.TB was found to have the highest sensitivity and this correlates well with studies of immunocompromised patients that have shown T-SPOT.TB to have better sensitivity than TST.[194-196]

Other studies compared IGRA and TST among contacts categorised into clinically defined gradients of exposure and sought to measure the agreement or discordance between the three. However, this is limited by differences in the degree and categorisation of exposure. The prevalence of positives was found to be similar for the most exposed groups but the TST had a greater prevalence of positives in the least exposed groups. Discordance was shown to be higher in persons with BCG vaccination, especially those vaccinated at age two years or older.[63;197;198]

Despite the variability in studies to date the most consistent finding has been the high specificity of IGRA. This is most likely due to the fact that IGRA use antigens that are not found in BCG or most non-tuberculous mycobacteria. To date there have been insufficient studies of IGRA performance among children, immunocompromised persons and the elderly.

4.7 Laboratory Safety

Levels of containment

The principal legal framework governing safety in relation to biological agents is contained in the following:

- The Safety, Health and Welfare at Work Act 2005
- The Safety, Health and Welfare at Work (General Application) Regulations 2007 (S.I. No. 299 of 2007)
- The Safety, Health and Welfare at Work (Biological Agents) Regulations, 1994 as amended in 1998 (S.I. No. 146, 1994, and S.I. No. 248 of 1998).

These give effect to the European Council, Biological Agents Directives.

Official copies of the legislation can be purchased from the Government Publications Sale Office, Sun Alliance House, Molesworth Street, Dublin 2. Tel. No: 01-6476000 or copies can be downloaded from www.irishstatutebook.ie.

The following containment measures in the Seventh Schedule (Safety Health and Welfare at Work (Biological Agents) regulations, S.I. No. 146, 1994) of the legal framework are compulsory:

- Extract air to the workplace are to be filtered using HEPA
- Access is to be restricted to nominated workers only
- There must be specified disinfection procedures
- Effective vector control e.g. rodents and insects
- Surfaces (bench and floor) impervious to water and easy to clean
- Surfaces resistant to acids, alkalis, solvents and disinfectants
- Safe storage of a biological agent and
- Infected material is to be handled in a safety cabinet (class 1 or class 2) or other suitable

containment.

Although the Seventh Schedule recommends the following points, the National TB Advisory Committee is proposing that these recommendations outlined below should be considered compulsory.

- The workplace is to be separated from any other activities in the same building
- The workplace is to be sealable to permit disinfection
- The workplace is to be maintained at an air pressure negative to atmosphere
- An observation window, or an alternative, is to be present, so that occupants can be seen and
- A laboratory is to contain its own equipment.

The decision to make this proposal has been influenced by looking at best international practice in relation to the items that are recommended. In the UK, the Control of Substances Hazardous to Health regulations 1994 (COSHH) implements the EC Biological Agents Directive. The Advisory Committee on Dangerous Pathogens (UK) (ACDP) originally published the *Categorisation of biological agents according to hazard and categories of containment (4th edition) 1995.*[199] This guidance has now been replaced by *The management, design and operation of microbiological containment laboratories, 2001.*[200] This publication complements the Health and Safety Commission's Health Service Advisory Committee's (HSAC) guidance on *Safe working and the prevention of infection in clinical laboratories and similar facilities*[201] and the ACDP publication *Biological agents: Managing the risks in laboratories and healthcare premises.*[202] Under this guidance the above recommendations are compulsory.

In the USA, laboratories performing functions at ATS level 2 or 3 must use Biosafety Level 3. Biosafety level 3 is very similar to Containment Level 3 (CL3) and the above recommendations are included as rules.[114;203] Finally, a CL3 Laboratory will fail to achieve accreditation with Clinical Pathology Accreditation (UK) Ltd if these recommendations are not in place.

Recommendation:

Recommendations in the Seventh Schedule of the S.I. No. 146/1994 Safety, Health and Welfare at Work (Biological Agents) Regulations 1994 should be interpreted as mandatory in relation to working with *M. tuberculosis* complex.

Transportation

The transport of infectious substances by road, rail, sea and air are each the subject of international regulation, whose whole description is beyond the remit of these guidelines. The relevant legislation in Ireland for transport by road is the "Carriage of Dangerous Goods by Road" Regulations 2007 Statutory Instrument No. 288 of 2007[204] and the 2007 ADR regulations.[205]

Specimens

Biological substances that have been correctly classified as UN No. 3373 which are packaged and marked in accordance with packaging instruction P650, are not subject to any other requirements of the 2007 ADR regulations. This implies that provided the correct packaging is used, there is no requirement for any ADR documentation or for the requirement to have specially marked and equipped vehicles or trained drivers. In summary, any method of transportation can be used to transport patient specimens by road once properly packaged and labelled.

Cultures

For transport purposes, pathogens are assigned to two categories, A and B. Category A includes the higher risk infectious microorganisms such as *M. tuberculosis* complex but only when it is present as a culture. Nevertheless, when cultures that are being transported by road are intended for **diagnostic** or **clinical** purposes (not research), they may be classified as infectious substances of Category B. Infectious substances in Category B shall be assigned to UN No. 3373 and the proper shipping name of *UN No. 3373*

is *"BIOLOGICAL SUBSTANCE, CATEGORY B"*. These are transported in accordance with ADR packaging regulations P650.

However, at the time of publication of this document, when cultures of *M. tuberculosis* complex are being transported by air or sea they must follow the International Air Transport Association (IATA) and the International Maritime Dangerous Goods (IMDG) regulations, that currently assign *M. tuberculosis* to Category A and they must be transported as *UN No. 2814 "INFECTIOUS SUBSTANCE AFFECTING HUMANS"* and packaged in accordance with packing instruction P620.[205] This is most easily achieved by using an approved and licensed courier.

M. tuberculosis complex outside the CL3 laboratory

It is worth noting that *M. tuberculosis* has been recovered from many body sites.[206-209] It follows therefore that appropriate risk assessments with regard to *M. tuberculosis* are carried out when processing samples for other pathogens in other parts of the laboratory. This is especially so when an extended incubation time is applied to recover fastidious pathogens. The observation that *M. tuberculosis* can be grown within six days on blood agar and that blood agar is at least as efficient as Lowenstein Jensen medium in recovering *M. tuberculosis* from respiratory and lymph node aspirates must be considered when performing risk assessment analysis for other procedures in the microbiology laboratory.[210] Respiratory specimens, in particular, may contain viable *M. tuberculosis* organisms and there are recommendations that these should be analysed in a CL3 facility.[199;211]

Recommendation:

All relevant legislation (national and international) for the transport and handling of specimens and cultures for tuberculosis should be strictly adhered to at all times.

Molecular testing

Tests involving molecular methods do not require CL3 practices after the organism has been rendered non-viable. This is normally achieved through the application of heat. Some systems have been shown not to render the cultures completely unviable and therefore a laboratory must be able to definitively state and demonstrate over time that any protocol in use for extracting mycobacterial DNA/RNA has succeeded in rendering the sample non-infectious before its removal from a CL3 facility. Methods which have produced consistent killing of all mycobacterial species tested are those by which the tubes were fully immersed in boiling water or within a forced-hot-air oven set at 100°C.[212-214]

Audit

There should be regular safety audits of the CL3 premises and processes.

4.8 Quality Assurance

Modern laboratory practice necessitates participation in both an internal and an approved external quality assurance scheme e.g. United Kingdom National External Quality Assessment Service (NEQAS). Quality assurance is a system that monitors and improves the efficiency and reliability of the laboratory service by paying attention to detail at every step. There are three phases to the process as follows:

Pre-analytical activities

- How the test is ordered
- Specimen collection procedures
- Transport to the laboratory
- Specimen handling and storage and
- Completeness of patient information.

Analytical activities

In the laboratory quality assurance procedures guide and monitor all related activities including:

- Instrument maintenance and operation
- Test reagents
- Personnel and
- Actual test performance.

Post-analytical activities

Work quality continues to be monitored in areas such as:

- Report sent to the appropriate party
- Timely reporting of data and
- Immediate reporting of positive results.

Essential components of a quality assurance programme are:

- Quality control
- Quality improvement
- Proficiency testing.

A quality control system is essential for the effective and systematic monitoring of the performance of bench work against established limits of acceptable performance. It ensures that the information generated by the laboratory is accurate, reliable and reproducible. Effective monitoring is carried out through regular audit at all levels in the process. Proficiency testing is essential and can be undertaken by participation in external quality control schemes such as NEQAS. Quality assurance is an essential component in achieving accreditation to ISO or CPA UK Ltd standards.

Recommendation:

Laboratories should participate in internal and external quality assurance schemes for all tests performed.