Mycobacterium diagnostics: from the primitive to the promising

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ABSTRACT

The field of clinical microbiology has been revolutionised by genomic and proteomic methods, which have facilitated more rapid diagnosis and characterisation of infection in many cases. In contrast, mycobacteriological evolution has tended to retain the traditional methods of smear microscopy for detection of acid-fast bacilli to indicate mycobacteria, along with culture, and in synergy with more modern molecular methods. Thus, efforts have been focused on reducing the time to diagnosis of infection, while increasing the amount of diagnostic information available, including more definitive speciation, and more rapid susceptibility test results. Although smear microscopy remains a mainstay for the laboratory-based diagnosis of mycobacterial infection, molecular testing has vastly reduced the time needed for identification of *Mycobacterium tuberculosis* in particular, when compared with traditional culture-based techniques. Molecular methods may also yield antimicrobial susceptibility results through testing for the most common resistance-inducing mutations to some of the antimicrobial agents of choice. However, the diversity of resistance mutations already characterised suggests that these currently-available molecular detection systems should be accompanied by culture-based susceptibility testing. This review compares the efficacy of microscopic, phenotypic, proteomic and genotypic methods available for mycobacterial diagnosis. The diversity of methods currently in use reflects the complexity of this area of diagnostic microbiology.

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Molecular biology.
Mycobacteriaceae.
Mycobacterium tuberculosis.
Spectrometry, mass, matrix-assisted laser desorption-ionization.
Tuberculosis.

Introduction

Mycobacteria are a heterogeneous group of acid-fast staining bacteria (AFB) that are generally classified as Gram-positive.[1] They include the causative agents of tuberculosis (TB), a disease that infects over eight million people each year with a fatality rate of over 15% worldwide.[2] In the UK and Ireland, recent rates of TB are similar at 12.3/100,000 and 9/100,000, respectively.[3,4] Rates have increased in the UK since 2002, primarily due to higher rates of TB among the non-UK-born population, which surpasses 1/1000 for certain subpopulations.[3] In addition, resistance to firstline therapy is increasing; and the total European (Including EU, non-EU and EEA member states) rate of multidrug resistance (MDR-TB) among all notified cases was 13.1% in 2011.[5] The prompt and accurate diagnosis of mycobacterial disease is particularly important in ensuring timely clinical management and in curbing any increase in MDR-TB.

Mycobacteria are loosely classified into one of two groups: *Mycobacterium tuberculosis* complex (MTC) and non-tuberculous mycobacteria (NTM) or *Mycobacterium* other than tuberculosis (MOTT).[6] The MTC species include *M. tuberculosis* (MTB), *M. bovis*, *M. africanum* and *M. microti*. The MOTT group is a large heterogeneous group of species that cause both pulmonary disease and extrapulmonary disease and that include *M. kansasii*, *M. avium*, *M. intracellulare*, *M. abscessus*, *M. marinum*, *M. ulcerans*, *M. leprae* and *M. scrofulaceum*. All MTC are classified as slow growing and can take up to 42 days to grow, whereas some of the MOTTs are fast growing by comparison, most notably *M. kansasii*.

As technology advances, more and more new species of mycobacteria that are potentially pathogenic to humans are being discovered. This, combined with increasing rates of antimicrobial resistance among well-characterised strains of mycobacteria, poses a challenge to accurate and prompt diagnosis.

Traditionally, the diagnosis of tuberculosis infection relies on a combined approach following clinical symptoms, namely a chest X-ray and laboratory testing. Currently in the clinical microbiology laboratory, diagnostic methods include microscopy, culture, identification and phenotypic susceptibility testing, and molecular methods.

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Preliminary mycobacterial screening methods

There are a number of initial screening tests used to rule-in a TB infection where a diagnosis is suspected on clinical grounds or following radiological findings. These include the tuberculin skin test and the interferon-gamma release assays (IGRA), and determining acid-fastness on a slide test.

Tuberculin skin test

The tuberculin skin test (TST), known as the Mantoux test, is one of few *in vivo* testing protocols involved in the initial screening of a TB infection. It involves an intradermal injection of purified protein derivate (PPD; tuberculin), which is a sterile preparation containing antigens from seven strains of *M. tuberculosis*.[7,8] Following a period of between 48 hours and 72 hours, the site of injection is examined and interpretation is made based on the size of induration (not the area of erythema). The principle of the TST is that the introduction of mycobacterial antigens induces an immune response (delayed-type hypersensitivity response) in those who have been previously exposed to mycobacteria.[9] Interpretation of the size of induration is detailed in Table 1.

It is important to note that the Mantoux test is non-specific; a positive reaction may result from previous BCG vaccination, MOTT infection (including from *M. avium* complex and *M. kansasii*) and latent TB.[10–12] Conversely, a negative TST result does not necessarily exclude active or latent TB infection. Many factors may result in a false negative including recent TB infection (where primary infection is within the last eight weeks), extremes of age, recent viral infection, recent live viral vaccination, illnesses (notably advanced HIV infection), and many others.[10,11] Therefore, the positive and negative predictive values of TST must be carefully considered in light of these influences.

Interferon-gamma release assay

An interferon-gamma release assay (IGRA) test is indicated when a positive TST is attained, where a TST is negative and when TB is strongly suspected, and prior to the administration of immunosuppressive medication to rule out latent TB. The premise of the IGRA is immunological; measuring T-cell interferon-gamma (INFγ) activity, either the concentration of INFγ directly from serum by enzyme-linked immunosorbent assay (ELISA; e.g., Quantiferon TB Gold In-Tube) or by enumeration of INFγ producing T cells by ELISA (e.g., TSPOT.TB).[11,13]

The IGRA test has shown to be more sensitive than TST for both active and latent pulmonary TB infections.[14] The value of IGRA testing for cases of active TB infection remains contentious;[13] however, a detailed meta-analysis of Quantiferon TB Gold In-Tube has shown a pooled sensitivity of 70% in cases of culture-positive TB.[14] The main reported advantages that IGRA has over TST are higher sensitivity, specificity and lack of subjectivity. Moreover, IGRA has been shown to be highly specific in cases of BCG-vaccinated patients. Specificity was 89% or greater across six studies of over 650 patients who had been BCG vaccinated compared to a mean specificity of 57% for the same cohort for TST.[13]

Public Health England recommends that IGRA testing not be used as a routine diagnostic tool for active TB, but for it to be considered to have a role in ruling out active TB, and that it may only assist a diagnosis in cases where mycobacteria remain unculturable, coupled with strong clinical findings in the form of chest X-ray or histological evidence.[15] However, the use of IGRA in detection of latent TB cases is as close to a gold-standard investigation as is currently routinely available, in the absence of a true gold-standard testing protocol.[7,15]

Table 1. Guidelines for interpretation of tuberculin skin test reaction.

Induration diameter	Considered positive for:
>5 mm	• HIV-infected individuals
	• Recent contact with individual(s) with infectious TB
	• Individual with fibrotic changes on a chest radiograph
	• Immunosuppressed individuals: organs transplant patients, patients on prolonged corticosteroid or TNFα antagonist therapies
>10 mm	• Individuals from TB endemic regions
	• Intravenous drug abusers
	• Staff in mycobacteriology laboratories
	• Individuals working and living in high-risk, high-population settings, including prisons, military confines etc
	• Individuals with high-risk underlying illness, including but not limited to diabetes, cancer, severe kidney disease
	• Children under five years old
	• Individuals including infants, children, adolescents exposed to adults in high-risk categories
>15 mm	• Individuals with no known risk factors for TB

Data obtained from ref 10.

Staining protocols

Arguably the most important step in the treatment and management of a case of TB, from a clinical and public health point of view, is the initial microscopic detection of mycobacteria in a sputum or other sample. Since the latter half of the 19th century, acid-fast staining has been the first step in the detection of mycobacteria.[16] It is a reliable, rapid and cost-effective firstline screen for the presence or absence of AFB that may be mycobacteria. The most widely used stains currently in use are Ziehl-Neelsen (ZN) and auramine fluorochrome stains.[3]

UK Standards for Microbiology Investigations outlines the methods of each of these stains, and the microscopic findings in positive and negative slides, along with potential controls that must be used to ensure a high level of

Table 2. A scheme for AFB reporting outlined by CDC.

Number of AFB seen per stated field (100x objective)	Proposed grade
0 AFB/100 fields	Negative (–)
1–9 AFB/100 fields	Actual number of AFB seen on whole slide
10–99 AFB/100 fields	1+
1–10 AFB/field in 50 fields	2+
>10 AFB/field in 20 fields	3+

Note: WHO states that the presence of at least one AFB in at least one sputum sample is a smear-positive pulmonary TB case.[19]

confidence in the result attained.[3] The grading of microscopy is subjective and can vary between microscopists. It is important, therefore, for a standard to be implemented within and between laboratories, similar to that outlined by the Centers for Disease Control and Prevention (CDC), as detailed in Table 2.

Sensitivity of ZN staining coupled with light microscopy is highly variable and can be as low as 20%.[17] The World Health Organization (WHO) recently advised that auramine staining with light emitting diode (LED) microscopy should replace ZN and light microscopy, and auramine and conventional fluorescence microscopy. The WHO states that LED microscopy is more accurate, less hazardous and cheaper than the ZN protocol, and the fluorescence microscopy of auramine-stained slides.[17] The use of LED microscopy increases accuracy of diagnostic microscopy by at least 5%, based on the results of the WHO Strategic and Technical Advisory Group for Tuberculosis.[17] Extrapolating from a 51% detection rate of culture-positive TB by direct microscopy for the UK in 2012, the sensitivity of microscopy still falls short of being ideal.[7] However, microscopy is still the most widely used laboratory screening method worldwide,[18] allowing rapid initiation of treatment and control measures where positive.

There are some caveats associated with AFB staining in general, including sensitivity (as previously outlined) and specificity. A positive AFB stain does not necessarily indicate an active TB infection, or indeed confirm a mycobacterial infection at all. Other acid-fast (and variably acid-fast) bacteria such as *Nocardia* species must also be considered with the interpretation of microscopy results. These downfalls of microscopy highlight the requirement of culturing systems to rule out any contaminant or false positives that may arise from, for example, the use of tap water (associated with *M. gordonae* contamination).[20]

Culturing systems

Following staining, laboratory confirmation of TB infection proceeds to isolation of the organism by a culture system. A decontamination step precedes culturing for samples from non-sterile sites, typically using NaOH.[21] A balance between decontamination of the sample of its non-mycobacterial flora and non-recovery of mycobacteria must be achieved, however. It has been suggested that a contamination rate of 2–5% is acceptable; as more strenuous decontamination methods may eliminate some mycobacterial species of interest.[6]

Culture systems involve either a solid or liquid medium. Solid media include Lowenstein-Jensen (LJ) medium (with or without glycerol) and other media including Middlebrook 7H10 and 7H11 (MB) formulations. Of the media currently available, LJ and MB are the recommended media of the European Respiratory Society, WHO, the American Thoracic Society and the International Union Against Tuberculosis (IUAT).[6,22,23] A number of studies have examined the sensitivity and specificity of LJ and MB, and results are variable.[24,25]

According to WHO, liquid culture systems are more sensitive and offer faster turnaround times both for isolation and direct sensitivity testing protocols than solid culture, and WHO therefore recommends liquid culture systems as giving improved standard of care for TB diagnosis.[26] Advantages of continuous monitoring liquid culture systems include being less labour-intensive, as they alert the user as soon as detectable growth occurs, they eliminate regular manual monitoring of solid media, reduce direct sensitivity testing (DST) time and increase sensitivity for isolation of MOTT species.[26] The disadvantages of these systems include expense, increased rates of contamination, and dependence on specific individual suppliers for the media, depending on the system utilised. Despite the importance of mycobacterial culture in the diagnosis of TB and MOTT infection, fewer than 70% of pulmonary TB cases are culture-confirmed in the UK.[3]

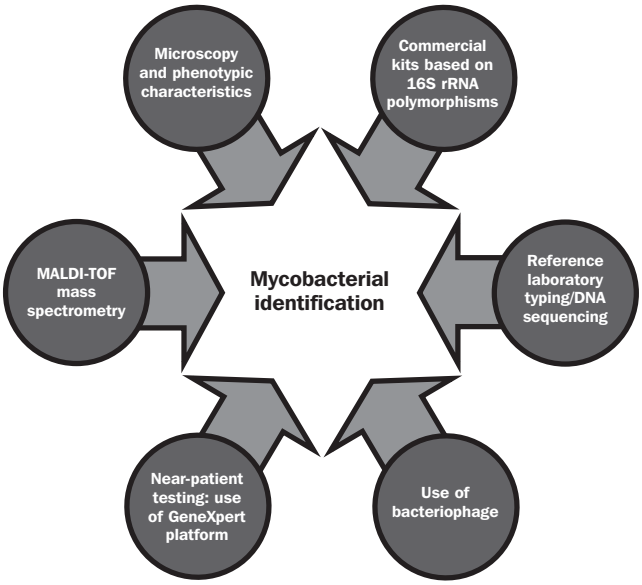

Fig. 1. Schematic representation of methods currently available for use in the clinical laboratory, either directly of indirectly through reference laboratories. The systems described are most often used in conjunction with one another, rather than in isolation.

Identification methods

The identification of the mycobacterial species responsible for infection usually occurs following successful culturing (an exception, however, being the near-patient method described below). Figure 1 outlines methods that can be used in isolation, but which are more commonly used in combination for mycobacterial identification. Following this, it is important that the bacterium is classified as MTC or MOTT. This step usually involves speciation also. Historically, this classification has been based on phenotypic appearance on culture coupled with growth patterns, including speed and physiological requirements in the form of biochemical testing panels.[27] One alternative strategy involves the use of phage directed against individual species. The use of phage-based tests for TB identification is mainly confined to developing countries, where more expensive assays are not widely available. One such method is described below.

Mycobacteriophage plaque assays

The FASTPlaqueTB assay (Biotech Laboratories, UK) relies on bacteriophage amplification technology. Specifically,

mycobacteriophage detects viable *M. tuberculosis* in decontaminated sputum samples. Following phage infection and multiplication within a viable *M. tuberculosis* strain and elimination of superfluous phage by virucidal treatment, the infected *M. tuberculosis* bacilli undergo lysis after proliferation of new phylogeny phage. The new phylogeny phage then infect *M. smegmatis* (which is fast growing, having a doubling time of 3–4 hours[28]), and the number of plaques present (higher than 20) indicates the presence of an active TB infection.[29] However, this method suffers from major disadvantages; for example, having a relatively low sensitivity, specificity, and a slow turnaround time compared with molecular methods.[29,30] However, it is still more accurate than AFB staining alone, and the improved assay to include detection of rifampicin resistance may increase its utility in countries where tuberculosis is endemic and where more expensive methods are not financially viable.[29,30]

Matrix-assisted laser desorption/ionisation–time of flight mass spectrometry

The use of matrix-assisted laser desorption/ionisation–time of flight (MALDI-TOF) mass spectrometry for the identification of bacteria and fungi has arguably been the single biggest innovation in clinical microbiology laboratories during the past decade. The two most popular systems are the VITEK MS (bioMerieux) and the MALDIBiotyper (Bruker Corporation). The two systems consist of a MALDI-TOF instrument and an automated database that analyses the mass spectra attained in the clinical laboratory. The databases in each case, Saramis and Biotyper, respectively, analyse the spectra and return a score based on the probability that the analyte belongs to a certain species or genus.[31] It should be noted that the means by which each system arrives at a potential identification differs.

The Bruker Main Spectrum analysis (MSP) involves a comparison of the mass spectrum attained for an isolate against a database of reference spectra from individual reference strains, which are derived from the collection of multiple replicate spectra, thereby eliminating, to a degree, intrastrain variances that may occur.[32] The bioMérieux system uses SuperSpectra to create the Saramis database and the Advanced Spectrum Classifier (ASC) is used to interrogate the database. SuperSpectra are the result of an accumulation of spectra for a particular strain, attained from both clinical and reference strains, which are often grown under different conditions. The ASC then compares the mass spectrum attained for a random clinical sample against the SuperSpectra, seeking peak presence/absence matching against all other species in the database.[32]

The use of MALDI-TOF for the identification generally relies upon an extraction step involving silica or zirconium beads, vortex mixing in the presence of ethanol, and further steps individual to each system.[33,34] The premise of MALDI-TOF is that a laser strikes a bacterial sample (either whole cell or following an extraction with formic acid, typically) in matrix, resulting in ionisation by charge transfer and desorption of the sample. The ionised molecules are then accelerated through an electric field in the time of flight tube, and meet a detector. The time of flight of the particles through this tube is based on the mass:charge ratio of the molecules, and repeated cycles of this result in a sequential series of detections, which is the mass spectrum attained at the end of the process.[32] The mass spectrum produced is essentially a protein fingerprint unique to the organism, as different bacteria express different surface proteins.

The majority of clinically significant mycobacterial ions are below 1 kDa; however, some including *M. avium* subsp. *Paratuberculosis* and *M. intracellulare* have characteristic peaks between 1.5 kDa and 5 kDa.[35] Early studies of the efficacy of MALDI-TOF in identifying mycobacteria involved using whole inactivated mycobacteria.[35,36] The main concerns when using whole cell mycobacteria include potential infectious exposure to laboratory personnel, and variable results depending on the glycopeptidolipid content of the mycobacterial cell wall,[37] along with poor reproducibility due to a litany of pre-analytical steps which are often not standardised.[33]

Inactivation protocols are necessary for most routine laboratories as the MALDI-TOF equipment is typically not located within the Category III containment facility. Inactivation protocols generally consist of a heat-inactivation step,[37–39] although it has been shown that ethanol inactivation in conjunction with centrifugation is sufficient.[36] Machen *et al*. showed that cell disruption is as effective as heat inactivation coupled with sonication for disruption of mycobacteria, also being reported as more sensitive (88.8% correct identification versus 82.2%, respectively).[34]

The sensitivity of MALDI-TOF for identification of mycobacteria to species level is variable, depending on the database and the success of extraction from the notoriously difficult-to-extract mycobacterial cell wall. It has been shown that when the extraction step is optimised the sensitivity can be higher than 90%.[40] The Bruker mycobacterial database 2.0 currently contains 131 mycobacterial species, while the Saramis database connected to the VITEK system contains 123 species; both systems have 13 species of MTC. In a recent study it was shown that bioMérieux's VITEK system, despite having fewer species in its database, showed a higher sensitivity of 94.4% and 87.4% using two different extraction protocols, compared to Bruker's 79.3% and 59.6%, respectively.[40]

MALDI-TOF identification of mycobacteria has proved to be accurate, rapid and less costly than molecular methods. The analytical sensitivities are variable, however, and are highly dependent on a number of pre-analytical and analytical variables.[33] A study by El Khechine *et al*. showed that various pre-analytical steps including heat inactivation coupled with cell disruption (achieved by use of Tween-20) increased the score attained.[39] Even with improved pre-analytical steps, the score attained still varies widely from study to study; from low scores at genus level to very high scores at species level.[33,35–37,40–42] This suggests that more work is needed to generate a standardised method for MALDI-TOF examination of mycobacteria, thereby also improving inter-laboratory reproducibility.

A study by Saleeb *et al*. showed a very high sensitivity utilising a Bruker MALDI-TOF; however, it is important to note that MALDI-TOF could not differentiate between certain groups of closely related mycobacteria.[37] Notably, *M. abscessus* and *M. massiliense* could not be discriminated on mass spectra alone. Subsequent studies have shown that MALDI-TOF analyses coupled with clustering analysis resulted in differentiation between these two closely related species.[43,44] MALDI-TOF was also unable to differentiate

between *M. mucogenicum* and *M. phocaicum* or between *M. chimaera* and *M. intracellulare*. Confident differentiation between these closely related species can only be achieved by MLST, qPCR methods and 16S RNA typing methods.[37,45–47]

Isolate identification using MALDI-TOF has many obvious advantages over molecular identification methods; it is faster, less expensive and is less labour-intensive. The major disadvantages of MALDI-TOF for mycobacterial identification include limitations associated with the commercial databases, namely the inability to distinguish between closely related species, and lack of standardisation for pre-analytical steps. However, these disadvantages may be overcome with extension of the commercial databases and the use of MALDI-TOF in collaboration with biostatistical analyses. In the future, genotypic identification of mycobacteria may be possible in the routine clinical laboratory using MALDI-TOF following post-PCR modifications (e.g., use of RNase T1 for G-specific cleavage) of 16S RNA and DNA, as described by Lefmann *et al*.[48]

Further developments, specifically involving electrospray ionisation mass spectrometry (ESI-MS) post-16S RNA PCR amplification of MTB and MOTT, has shown huge potential as an accurate means of mycobacterial speciation and also as a determinant of antimycobacterial agent resistance.[9] The culmination of the molecular and proteomic power of MALDI-TOF as a tool could result in rapid, accurate identification to strain level along with the prediction of antimicrobial susceptibilities, thereby greatly improving patient outcome in cases of mycobacterial infection. However, the use of MALDI-TOF for the identification of mycobacteria also has its critics, with at least one suggesting that the identification accuracy achievable currently with genetic approaches is out of the reach of MALDI-TOF technology.[50] It is evident that more research is needed to ascertain the true power of MALDI-TOF technology as a diagnostic tool for this genus.

Molecular-based identification

In the 1980s, the use of DNA-based techniques revolutionised the identification and strain determination of microbes, including highly conserved mycobacteria, with DNA relatedness between species of the MTC of 85–100%; *M. microti* and *M. bovis*, for example, being responsible for the lower levels of relatedness.[51] The highly conservative nature of the MTC can be attributed to the rDNA operon which transcribes internal transcribed spacer (ITS) regions along with rRNA, resulting in near homology between species of the MTC, with no nucleotide differences occurring between pairs of 16S–23S rDNA ITS sequences for MTC.[52] However, this 16S–23S rDNA analysis has proved to be useful in speciating MOTT. Notably, studies have shown that the MAI group (*M. avium-intracellulare*) cluster has a number of distinct sequevars (regions of ITS that differ by at least two base pairs) allowing for reliable speciation and in some cases determination of subspecies; *M. avium* has at least four sequevars, allowing for the discrimination of at least four subspecies,[53] although this level of discrimination is not routine when diagnosing mycobacterial infection.

Clinical laboratory diagnosis of mycobacterial infection through rRNA-based PCR has typically involved visualisation of products through the use of adjunctive technology such as the GenoType CM/AS (Hain Lifescience, UK) system. The GenoType *Mycobacterium* CM/AS (Common Mycobacteria/Additional Species) DNA strip assay exploits the polymorphisms that exist in the 23S rRNA of mycobacteria and allow for rapid identification of 25 of the most commonly encountered mycobacterial species using the GenoType CM, and 19 additional MOTT species using the AS extension kit. The principle of the assay is dual reverse hybridisation coupled with PCR, specifically that a hybridisation strip with immobilised probes specific for certain mycobacterial species is directed against the PCR products of the 23S rRNA gene.[54] The GenoType CM/AS system has been shown to have high sensitivity in numerous studies, ranging from 88–97% and specificity as high as 100%.[55–59] The main drawbacks of this system appear to be cost (Makinen *et al*.), a lack of probes, particularly for some MOTT species;[60] also that it appears to be less sensitive than other commercially available kits such as the Gen-Probe Accuprobe *Mycobacterium* system (Gen-Probe, San Diego, USA),[58] and Inno-LiPA kits (Innogenetics, Belgium).[61]

The basis of the Inno-LiPA kit is very similar to that of the GenoType CM/AS kit, involving PCR of the 16S–23S rRNA spacer region and reverse hybridisation of the amplicons, using strips that can simultaneously identify 14 species (closely related species are grouped; e.g., *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. malmoense*, *M. haemophilum*).[62]

The genome of *M. tuberculosis* H37Rv was first sequenced and annotated in 1998.[63] This extensive study, subsequently updated in 2002, showed that the genome comprises 4.4 million base pairs and around 4000 genes. The study also includes a comprehensive functional classification of these 4000 genes.[63,64] There are now reference laboratories and specialist facilities that can conduct whole-genome sequencing (WGS) on request. Although WGS is expensive, it is the most accurate method of identification of mycobacteria.[65] Nonetheless, it is not a routine method and unlikely to become so in the near future.

Susceptibility testing and resistance mechanism determination

Tuberculosis treatment is empirical after diagnosis in most cases owing to the time taken for susceptibility test results to be available. The rates of multidrug-resistant (MDR-) and extensively-drug-resistant (XDR-) TB are increasing, making treatment efficacy less certain. The most commonly used firstline antimycobacterial agents are rifampicin, streptomycin, isoniazid, ethambutol and pyrazinamide. Rifampicin resistance is often an indicator of MDR, as the most commonly encountered MDR pattern shows resistance to rifampicin and isoniazid,[66,67] and up to 91% of rifampicin-resistant MTB have dual resistance to isoniazid.[68]

One study reported that three MTB isolates shown to be phenotypcially rifampicin-sensitive and isoniazid-resistant were associated with treatment failure subsequently traced to a *rpoB* gene mutation that was not expressed *in vitro*, thereby indicating the need for genotypic resistance determination in cases of substantiated rifampicin resistance.[69] It has been reported that 95% of the mechanisms giving rise to rifampicin resistance are due to modifications of *rpoB*, of which many have been found,

including single-nucleotide polymorphisms, deletions, insertions and missense mutations.[69,70,71]

The primary mechanism of resistance to isoniazid is *katG* mutation (the gene coding for catalase-peroxidase, whose action is required to activate isoniazid). Mutations in *katG* are seen in up to 60% of isoniazid-resistant isolates, and the *Ser315Thr* mutation has been the most commonly encountered mutation of the gene. However, the mechanisms of isoniazid resistance are diverse and many of these have been described, including *inhA* mutation, which is the most commonly found of the other mutations that confer resistance.[69,72–74]

Similarly, many resistance mechanisms have been demonstrated for pyrazinimide, ethambutol and streptomycin. The most common resistance-conferring mutations to date include mutation of the gene coding for pyrazinamidase, the mycobacterial enzyme that converts the pro-drug pyrazinimide into its active form, and mutation is found in up to 97% of pyrazinamide-resistant strains.[75,76] Among streptomycin-resistant mycobacteria, the mechanisms detected have been diverse and include mutations in genes encoding 16S rRNA, ribosomal proteins and other mechanisms that have not been well characterised.[76–79] Mutations in *embB*, which codes for an arabinosyl transferase, are most commonly associated with ethambutol resistance and have been found in up to 65% of ethambutol-resistant strains.[76]

Phenotypic methods of antimycobacterial drug susceptibility are slow and may lack reproducibility. It is also noteworthy that phenotypic methods may not be sufficiently sensitive to detect low-level resistance and may not correlate with *in vivo* susceptibility to the drug.[80] Modifications of molecular identification kits have allowed for rapid drug susceptibility results. Commercial kits such as the GenoType MDRTBplus (Hain Lifescience) assay and the Inno-Lipa systems have been modified to include a detection method for resistance to certain antimycobacterial agents. The premise of the GenoType MTBDRplus assay is similar to the GenoType CM/AS system previously described. It has been reported as being capable of detecting rifampicin, isoniazid and multidrug resistance.[81–83]

The detection of resistance is based on *rpoB* and *inhA* gene mutations for rifampicin and isoniazid, respectively.[84] Reported sensitivities of resistance gene detection have ranged from 81.8%[85] to more than 90%.[82,83] The GenoType MTBDRplus has been recommended by WHO for use in endemic areas with high levels of resistance. The WHO expert group found that the GenoType MRBDRplus was 30% less expensive than conventional direct sensitivity testing (DST), had a faster turnaround time and had increased sensitivity and specificity compared to DST.[85] The other system recommended by the WHO expert group was the Inno-Lipa Rif.TB system (Fujirebio, formerly Innogenetics).[85] This system can detect rifampicin resistance when mutations occur in *rpoB*.[86] However, the literature suggests that the Inno-Lipa RIF.TB system falls short of the MTBDRplus assay due to it being restricted to rifampicin resistance detection, more expensive, and its inability to identify MTB and rifampicin resistance from stained smears and paraffin wax-embedded blocks.[85,87]

Reports of the efficacy of these molecular detection systems are affected by their ability to detect the most common mutations in the regions where they are tested. For a system to be able to detect all mutations that may give rise to antimycobacterial resistance, a very wide range of loci would need to be targeted. One practical means to achieve such a system may be through the use of a multiplex PCR with primers designed to target the most frequently encountered mutation in a geographical area,[88] or using a series of primers directed against the most commonly encoutered mutations that confer resistance.[89] Other options include the use of a microarray with probes specifically designed to target the most common mutations in a certain population. Microarrays have been described that have shown efficacy in determining resistance against first- and second-line antimycobacterial agents, especially in cases of MDR.[90,91]

Near-patient testing for detection of mycobacteria

Near-patient testing for MTB, using Cepheid's Expert TB/RIF system (Cepheid, CA, USA), has gained widespread popularity and the support of WHO for testing in countries of low resource and high incidence of TB.[92] This test can simultaneously detect the presence of sequences specific for MTB and also for resistance to rifampicin in a closed hemi-nested real-time PCR system. The test amplifies the *rpoB* gene of *M. tuberculosis*. The assay is rapid, with a turnaround time of less than two hours; it has shown to have a sensitivity ranging from 72.5% for smear-negative, culture-positive samples, to up to 100% sensitivity in smear-positive, culture-positive specimens.[71,86,93] The specificity has shown to be as high as 100% both for MTB identification and rifampicin resistance detection, and the assay has been reported to have a limit of detection of 131 colony-forming units (cfu)/mL.[71,93] Owing to the closed nature of the system, there are fewer bioaerosols produced than with conventional AFB smear staining, perhaps suggesting its potential utility as a bedside test.[94] Bedside testing remains hazardous, however, especially for MTB[95] and this hazard may preclude the use of such a system in the near-patient setting.

Conclusions

In the clinical microbiology laboratory, we have seen traditional, subjective techniques such as rate of growth, appearance on culture, and biochemical tests to identify mycobacteria largely replaced by genomic and proteomic methods that allow for comparatively rapid and accurate identification to species level. However, there is still no 'one size fits all' *Mycobacterium* diagnostic system.

Despite the relatively low sensitivity and the inherent subjectivity of microscopy, this remains the most widely used primary laboratory test for the detection of a mycobacterial infection,[18] as it offers the clinician a rapid indication of AFB status. The prioritisation of subsequent steps and platforms depends wholly on the requirements, means and clinical situation for individual laboratories.

After microscopy, culturing is arguably still the most important step in mycobacterial diagnostics, notwithstanding the high proportion of cases that are not culture confirmed.[3] The best returns from culturing systems are seen with

appropriate sample treatment steps, media, supplements and growing conditions. Many of the more sensitive and accurate platforms, both genomic and proteomic, require sufficient bacterial cell density to be functional or useful.[39] It is also important to note that the isolation of mycobacteria is still pivotal for fulfilling the criteria of a definitive diagnosis of tuberculosis.[18]

The importance of mycobacterial speciation can be seen in the bearing it has on proper management and treatment of a patient. For example, there is no need for an immunocompromised patient who has contracted an opportunistic MOTT infection to be isolated, and their treatment will differ from a TB case. Equally, a patient with MDR-TB needs to be isolated and will require tailored therapy.[96] Resistance determination of mycobacteria is thus important for patient management and treatment. The platforms available for susceptibility determination, both culture-based and molecular methods, each have advantages and caveats. It appears that, to ensure reliable determination of sensitivities, a combination of culture and molecular systems is currently required until the deficiencies associated with molecular methods have been addressed.

Finally, proteomic profiling has shown to be accurate, relatively inexpensive in a high-throughput laboratory, and reliable in identifying a broad spectrum of mycobacteria.[36] However, MALDI-TOF requires further study in order to determine its discriminatory potential regarding susceptibility testing and epidemiological typing, and to overcome the described deficiencies of the technology that relate specifically to mycobacteria.

The current gold standard for identification remains in genomics.[50] These systems are expensive and thus out of the reach of under-resourced laboratories. This is why mycobacteriophage assays and other low-cost systems that allow identification are unlikely to be disregarded, notwithstanding their low sensitivity rates, in low-resource settings. □

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