The past, present and future of tuberculosis diagnostics techniques

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ABSTRACT

According to a recent World Health Organization report, tuberculosis (TB), an ancient disease with an enormous global impact, still ranks in the top ten causes of death worldwide. Pulmonary TB, where patients show symptoms like cough, fever, night sweat and weight-loss is highly contagious and poses serious public health concerns. If diagnosed in a timely manner nearly all patients infected with TB can be cured. However, TB remains one of the world’s big healthcare-related challenges since treatment usually requires daily intake of multiple antibiotics for a prolonged period of six months. As such patient compliance, as well as tolerance to this multi-drug therapy, poses a big issue in many cases, resulting in relapse or development of drug-resistance. The increase in resistance to the different front-line TB drugs has led to the spread of multi-drug resistant tuberculosis (MDR-TB) and further prevents TB control. MDR-TB creates a higher burden on clinical management requiring prolonged treatment, and monitoring of adverse side-effects from alternate therapy. In addition, presence of co-morbidities (HIV, immunocompromised status, diabetes) can further complicate cure. Despite a lot of research, the diagnostic testing and the epidemiological investigation to control the spread of TB still require significant resources of clinical microbiology laboratories as well as public health services.

This review article provides a brief overview of the past, present and future of the different diagnostic technologies used in tuberculosis. It covers the time-intensive culture-based technologies, along with the newer molecular technologies that have revolutionised TB diagnosis with faster turn-around-time. This review article also describes the technologies that rapidly determine the drug susceptibility or resistance of the bacterium, Mycobacterium tuberculosis, the causative agent of TB; and the technologies of molecular typing that help in an outbreak or prevent further spread of the disease. It discusses research that has contributed to further understanding of the biology of various strains in the Mycobacterium tuberculosis complex, and their prevalence in humans globally and in New Zealand. Finally, the emerging technology of whole genome sequencing of Mycobacterium tuberculosis and its possible applications in TB diagnosis is discussed.

Keywords: Tuberculosis, epidemiology, New Zealand, TB drugs, drug-resistance, diagnostic tools, TB typing, application of WGS.

Epidemiology of tuberculosis

Highly infectious tuberculosis (TB) is primarily a pulmonary disease that has been a human scourge for many centuries. TB was known in the 18th and 19th centuries as consumption, since the disease “consumes” or causes drastic weight loss of the afflicted individual over the course of the disease progression. The onset of symptoms in pulmonary cases is usually weight loss, lethargy, night fevers and a cough – which is usually productive and may include visible blood. Coughing expels droplets of respiratory secretions containing the bacteria into the surrounding air, which can be inhaled to infect others making TB highly contagious. While primary infection is generally confined to the lower respiratory tract, in some patients the bacteria can spread to other sites via the blood stream, leading to disseminated or miliary tuberculosis, which if undetected and untreated, can lead to death.

Tuberculosis can also manifest clinically by affecting various other extra-pulmonary sites. Tuberculosis has claimed many lives over the centuries including Jane Austen in the 18th century, Frederic Chopin in the 19th century, and New Zealand’s very own Katherine Mansfield in the 20th century. TB continues to remain a major challenge for definitive global prevention and cure and continues to take the lives of millions in the developing world, even in the 21st century. TB is one of the top 10 causes of death worldwide (1). It ranks above HIV/AIDS in the top 10 causes of death globally due to a single infectious agent (1). In 2017, TB was responsible for an estimated 1.3 million deaths among HIV-negative people (1). The global emergence of antimicrobial resistance as well as co-morbidity with HIV/AIDS poses additional challenges for tuberculosis control. On a positive note, the World Health Organization (WHO) set a target to “End TB” by 2035 considering the development of better therapeutic interventions like new antimicrobials, better diagnostic methods through more advanced technologies and better preventative, for example, the improvement of vaccines through better understanding of disease pathogenesis (1).

According to the latest ESR report, the 2015 notification rate of tuberculosis in New Zealand was 6.2 per 100,000 (2). Hence, New Zealand has a low burden of tuberculosis compared to the nations in the Indian subcontinent, South East Asia, and Africa which share 84% of the global burden of TB according to the WHO report for 2018 with >10,000 estimated TB cases per year (1).

Mycobacterium tuberculosis, the causative agent of tuberculosis

In 1882 Dr Robert Koch shared his discovery of Mycobacterium tuberculosis or tubercle bacillus, the causative agent of tuberculosis with the scientific community (3). Thus he presented his ground breaking postulate in microbiology, famously known after him as the Koch’s postulate, where he showed a causal relationship between a microorganism and...
a disease. He won the Nobel Prize in Physiology or Medicine in 1905 for this discovery.

*M. tuberculosis* is quite different from other bacteria because of its slow growth rate when cultured. Specimens from patients suspected of infection with *M. tuberculosis* are cultured for a maximum of 6 weeks, if the bacterium does not grow. Another special characteristic of *M. tuberculosis* is its thick and waxy outer layer that makes it impervious to usual staining techniques used for other bacterial detection and groups it under “acid-fast bacillus” (AFB) as a result (4).

Currently, the genus *Mycobacterium* consists of more than 200 species (5, 6). *Mycobacteria* can be divided into the following groups based on the clinical manifestation of the disease in humans and growth characteristics *in vitro* - *Mycobacterium tuberculosis* complex (MTBC) causing tuberculosis in humans, *Mycobacterium leprae* causing leprosy; *Mycobacterium ulcerans* causing Buruli ulcer; and those referred to as nontuberculous mycobacteria (NTM) (6) that can be sub grouped as rapid growers (grows in <7 days) and slow growers (grows in >7 days). MTBC causing tuberculosis is comprised of human adapted pathogens and animal adapted pathogens (7). The human adapted ones are *Mycobacterium tuberculosis sensu stricto*, *M. africanum* isolated mostly from West Africa (8) and *M. canetti* isolated only from the Horn of Africa (9). Among the various animal adapted pathogens, *M. bovis* cause tuberculosis in cattle. In the late 19th and early 20th centuries *M. bovis* caused around a quarter of all human cases of tuberculosis when humans ingested contaminated dairy products (10). In spite of pasteurization reducing the risk of transmission of *Mycobacterium bovis* from infected cattle to humans from the 1940s, *M. bovis* transmission from animals to humans has been reported in New Zealand in recent times (11). Other animal adapted pathogens include *M. caprae* causing tuberculosis in goats, *M. microti* in rodents like voles, *M. orygis* in antelopes, *M. pinnipedii* in seal and sea lions, *M. mungi* in banded mongooses, *M. suricattae* in meerkats, the dassie bacillus in hyrax and the chimpanzee bacillus (7).

*M. tuberculosis* has a long history of co-evolution with its human host (12, 13). Phylogenetic analysis of MTBC culture isolates based on phylogenetic markers, large sequence polymorphisms (LSP) or regions of difference (RD) showed that *Mycobacterium tuberculosis* comprises several phylogeographical lineages (Figure 1a) (14). Among *Mycobacterium tuberculosis* clinical isolates, the main human-adapted strain lineages are classified into Lineages 1–7 according to their geographic distribution. Indo-Oceanic or Lineage 1 is known as the “ancestral” or “ancient” strain and the rest of the “modern” strains are East Asian or Lineage 2, East African-Indian or Lineage 3, Euro-American or Lineage 4 and Ethiopian or Lineage 7 (14). Lineages 1–4 comprise the majority of human-adapted strains which are responsible globally for human TB cases, while West African 1 or Lineage 5 and West African 2 or Lineage 6 are restricted to West Africa and are traditionally known as *M. africanum* West Africa 1 and 2, respectively. These seven phylogeographic lineages are associated with particular geographic regions and differ among others, in virulence, biological fitness and propensity to acquire drug resistance (7).

![Figure 1. The global population structure and geographical distribution of *M. tuberculosis*.](image)

(a) Large scale polymorphism (LSPs) define a global phylogeny for *M. tuberculosis*. The names of the lineage-defining LSPs or regions of difference are shown in rectangles. The geographic regions associated with specific lineages are indicated.

(b) The six main lineages of *M. tuberculosis* are geographically structured. Each dot corresponds to 1 of 80 countries represented in the global strain collection. The colours of the dots relate to the six main lineages defined in Fig. 1a and indicate the dominant lineage(s) in the respective countries.

Lineage 4 is the most widespread lineage of *M. tuberculosis* causing human tuberculosis globally (Figure 1b). A more recent study by Gagneux and his collaborators further investigated the global distribution of *M. tuberculosis* lineage 4 (15) using a wider collection of clinical isolates. In this study, employing molecular whole genome sequencing based phylogenetic techniques on the global collection of lineage 4, the authors inferred that the global success and spread of lineage 4 is a consequence of both biological and social phenomena. Furthermore, they also show that the lineage 4 of *M. tuberculosis* is genetically diverse. Based on the phylogeography of lineage 4, the authors deduced a distinction between globally represented “generalist” sublineages and geographically restricted “specialist” sublineages within lineage 4 (15).

A collaborative study was undertaken in 2012 to ascertain the distribution of MTBC lineages in New Zealand and also to examine whether particular lineages correlate with patient ethnicity, country of origin and multi drug resistant tuberculosis (MDR-TB) (16). In this study, techniques similar to that used in the paper by Gagneux and co-authors in 2006 (14), i.e. large sequence polymorphism (LSP) and single nucleotide polymorphism (SNP) analyses, were applied on all representative laboratory confirmed *M. tuberculosis* stains isolated in New Zealand in 2010 and 2011. This study showed that New Zealand *M. tuberculosis* isolates are dominated by lineage 4 (Figure 2) (16).

In this study, it was found that among patients who were immigrants to New Zealand, the predominant lineages corresponded to highly prevalence lineages in the country of origin while lineage 4 was predominantly isolated in New Zealand-born individuals (Figure 3).


**Figure 3.** Relative abundance of phylogenetic lineages according to (A) country of origin and (B) ethnicity. (MTC = *M. tuberculosis* complex). The numbers inside the bars represent the lineages. Reference: Yen S, Bower JE, Freeman JT, Basu I, O’Toole RF. Phylogenetic lineages of tuberculosis isolates in New Zealand and their association with patient demographics. *Int J Tuberc Lung Dis* 2013; 17(7): 892–897. Reprinted with permission of the International Union Against Tuberculosis and Lung Disease. Copyright © The Union.
It was also noted in the same study that lineage 2 predominantly accounts for MDR-TB cases in New Zealand from 2002 to 2011 (Figure 4) (16). This is consistent with reports showing the Beijing sublineage belonging to “ancient” lineage 2 are more virulent, have better transmission capability and are MDR-TB isolates (17).

Anti-tuberculous therapeutics and emergence of resistance to anti-tuberculous drugs

Most bacterial infections are cured by a short course (a week or two) of antibiotic monotherapy. In contrast, an abundance of scientific evidence shows better outcomes for tuberculosis with a long-term and multidrug therapeutic approach, due to the slow growth and dormancy of a sub-population of MTBC (18). It takes at least 6 months to successfully treat uncomplicated drug-sensitive pulmonary TB. For a *M. tuberculosis* isolate which is fully susceptible to first line drugs, the regimen prescribed during the first two months consists of a daily dose of two bactericidal drugs, isoniazid and rifampicin, along with ethambutol which inhibits actively multiplying bacilli and pyrazinamide, a bacteriostatic agent that inhibits semi-dormant bacilli located in acidic environments such as TB lesions. After the intensive phase of the first 2 months with a daily dose of these 4 drugs, the sterilising phase in the following four more months comprises a daily dose of isoniazid and rifampicin. This long-term therapy with the combination of “first line drugs” has been shown to achieve bacteriological eradication (Table 1) (19).

Drug resistant TB results from inadequate or incomplete treatment that selects for drug-resistant bacilli (acquired resistance), and those resistant organisms can be transmitted to other individuals (primary resistance). Second-line drugs are used to replace the ineffective first-line drugs in complicated cases of drug resistant TB. The second line drugs are more costly and can lead to more adverse side effects. In case of multi-drug resistant tuberculosis [MDR-TB, defined as being resistant to both isoniazid (INH) and rifampicin (RIF)] the first two treatment options undertaken are with the higher generation fluoroquinolones (FQ), such as moxifloxacin or levofloxacin and one of the second line injectable drugs (SLIDs) which includes aminoglycosides and the cyclic peptides (20). Other drugs can be used according to the WHO guidelines (20). Details about drugs commonly used in treatment for drug-sensitive and drug-resistant tuberculosis discussed above, their mode of action and the mutations commonly implicating resistance are compiled in Table 1.

Drug resistance is concerning, as it requires more complexity with regards to the length of treatment, monitoring of adverse side-effect of the toxic second-line drugs and the cost of the treatment. An increase in multi-drug resistant TB (MDR-TB) has added to the global concern of tuberculosis. New Zealand has a low burden of MDR-TB at an average annual rate of 1.2% among culture-positive TB cases (2). WHO define extensively drug-resistant (XDR) TB as a multi-drugresistant tuberculosis that is also resistant to any one of the FQs and one of the SLIDs. New Zealand has only one recorded laboratory-confirmed case of an XDR-TB in 2010 (21).

The primary diagnostic tool used in a basic microbiology laboratory is smear microscopy of respiratory specimens predominantly induced sputum, with staining for acid-fast bacilli (AFB). This is simple, rapid and inexpensive but lacks sensitivity and varies greatly based on the AFB burden in sputum, with 1000–10,000 colony-forming units (CFU)/ml required for reliable detection (22). Culture of respiratory specimens is considered the gold standard for detection of pulmonary tuberculosis and requires only 10 CFU/ml to be positive in liquid culture (23). However, culture of *M. tuberculosis* must be performed in a high containment laboratory and requires expensive equipment. Solid medium-based cultures like the egg-based Löwenstein–Jensen (LJ) medium have a good specificity but require 3–6 weeks to grow (24).

In the recent years, the capacity for culture-based TB diagnosis has improved due, in part, to the new policy recommendations of the WHO (25) on the use of liquid-based culture medium, for example, the modified Middlebrook 7H9 broth base Mycobacteria Growth Indicator Tube (MGIT) from Becton Dickinson. Irrespective of smear results, which give an indication of the bacterial burden, the mean time to detection in the MGIT broth is 15.3 days for all mycobacteria (26). According to the WHO policy guidelines for any positive liquid cultures, all refer to mycobacterium tuberculosis. To differentiate and use a rapid and affordable method for the identification of the mycobacterial species in order to differentiate *M. tuberculosis* complex from non-tuberculous mycobacteria (NTM) (25). This differentiation is predominantly based on a secreted protein called the MPT64 that is released during the growth of *M. tuberculosis* complex in culture medium.

Several simple and useful immunoassays have been developed based on the detection of this MPT64 antigen in the culture medium (27). In the reference mycobacterial laboratory in LabPLUS the Becton Dickinson (BD) MGIT TBC (MTBC) Identification Test Rapid test, which detects the protein MPT64 in liquid or solid cultures is used for the rapid identification of *M. tuberculosis* complex. However, in the period following the adoption of BD MGIT TBC Identification Test, six MGIT culture isolates gave a negative result when this ICT was used for detection of MTBC. Since there was a high suspicion of TB in these 6 cases, the isolates were tested further with Xpert MTB/RIF PCR targeting the rpoB gene and were found positive for MTBC. These 6 isolates were further analysed to address the discrepancy in result obtained from the two tests. On sequencing the mpt64 gene, which codes for the MPT64 antigen, mutations were detected in the mpt64 gene in these false-negative cases due to deletion, insertion or substitution of bases. These mutations prevented the secretion of the MPT64 antigen in the culture medium and consequently prevent its detection by the MGIT TBC Identification rapid test (28). In spite of this constraint, this ICT is easy-to-use, rapid and affordable. Hence an algorithm has been implemented subsequently at the mycobacteriology lab in LabPLUS. If there is a high clinical suspicion of TB, a MGIT culture with a negative ICT result is further run through Xpert MTB/RIF PCR (with a different target) to rule out the presence of MTBC in the liquid MGIT culture (29). This presents the misidentification of tuberculosis from a MGIT culture.

Figure 4. Relative distribution of multidrug-resistant isolates of the Mycobacterium tuberculosis complex, 2002-2011 with respect to genetic lineage.

### Table 1. Anti-tuberculous drugs.

<table>
<thead>
<tr>
<th>Drug name (abbreviation)</th>
<th>Class of drugs</th>
<th>Mode of action</th>
<th>Mutation in gene(s) conferring resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin (RIF)</td>
<td>Rifamycin derivative</td>
<td>Interferes with RNA synthesis by binding to the β subunit of the RNA polymerase</td>
<td>rpoB</td>
</tr>
<tr>
<td>Isoniazid (INH)</td>
<td>Pyridine [a pro-drug that requires activation by the catalase/peroxidase enzyme KatG, encoded by the katG gene, to exert its effect]</td>
<td>Inhibits synthesis of mycolic acids through the NADH-dependent enoyl-acyl carrier protein (ACP)-reductase, encoded by inhA</td>
<td>katG, inhA</td>
</tr>
<tr>
<td>Ethambutol (EMB)</td>
<td>Ethylenediamine</td>
<td>Inhibition of arabinogalactan biosynthesis in cell wall</td>
<td>embB</td>
</tr>
<tr>
<td>Pyrazinamide (PZA)</td>
<td>Pyrazine; an analogue of nicotinamide</td>
<td>Converts pyrazinamide to pyrazinoic acid, which disrupts the bacterial membrane energetics inhibiting membrane transport</td>
<td>pncA</td>
</tr>
<tr>
<td>Moxifloxacin/Levofloxacin</td>
<td>Fluoroquinolone (FLQ)</td>
<td>Inhibition of topoisomerase II or DNA gyrase involved in DNA synthesis</td>
<td>gyrA, gyrB</td>
</tr>
<tr>
<td>Kanamycin/Amikacin</td>
<td>Aminoglycoside (second line injectable drugs; SLIDs)</td>
<td>Inhibition of protein synthesis</td>
<td>rrs, eis</td>
</tr>
<tr>
<td>Capreomycin/Viomycin</td>
<td>Cyclic peptide (second line injectable drugs; SLIDs)</td>
<td>Inhibition of protein synthesis</td>
<td>rrs</td>
</tr>
</tbody>
</table>

The phenotypic drug susceptibility testing (pDST) of MTBC can take 2 to 4 weeks, since it is constrained by the slow growth characteristics of *M. tuberculosis*. Therefore, it is time-consuming, requires advanced and sophisticated laboratory infrastructure, qualified staff and strict quality assurance mechanisms. Moreover pDST can be challenging and in resource-limited areas it can be cost-prohibitive. Yet, it is still considered the gold standard for drug resistance detection. Phenotypic DST uses critical concentrations which is defined as the lowest concentration of an anti-TB drugs in vitro that will inhibit the growth of 99% of phenotypically wild type strains of MTBC. Critical concentrations of anti-TB agents is used to determine the susceptibility or resistance of a culture of MTBC in resource-limited areas it can be cost-prohibitive. Yet, it is still considered the gold standard for drug resistance detection. Phenotypic DST uses critical concentrations which is defined as the lowest concentration of an anti-TB drugs in vitro that will inhibit the growth of 99% of phenotypically wild type strains of MTBC. Critical concentrations of anti-TB agents is used to determine the susceptibility or resistance of a culture of MTBC. Critical concentrations of anti-TB agents is used to determine the susceptibility or resistance of a culture of MTBC.

To address the growing global need for prompt and accurate detection of tuberculosis, the use of genotypic drug susceptibility testing (gDST) took off with the introduction of the Cepheid Xpert MTB/RIF test. It is a commercial, automated, real-time PCR based-technology which detects the presence of MTBC as well as RIF resistance (30). The introduction of Xpert MTB/RIF test was a “game-changer” in TB diagnosis since the result is available with minimal hands-on technical time and less than 2 hours of PCR from direct respiratory specimens like induced sputum. Cepheid Xpert MTB/RIF assay, endorsed by WHO, has become a valuable first-line test for the detection of *M. tuberculosis* and rifampicin resistance, especially in resource-constrained countries. More than 95% of RIF resistant mutations are associated with mutations in the *rpoB* gene and the majority of the mutations in *rpoB* gene are found within an 81 base pair (bp) RIF-resistance determining region (RRDR). Hence Xpert MTB/RIF PCR technology integrates this 81bp mutational hot-spot in the *rpoB* gene, and thus not only detects the presence of MTBC DNA in a clinical specimen but also whether there is a RIF resistance-associated mutation in the RRDR hot-spot resulting in presumptive MDR-TB. Mycobacterial resistance to RIF mostly occurs together with resistance to INH and hence resistance to RIF is considered as a surrogate marker for MDR-TB (31).

However, during routine diagnostic testing at LabPLUS, the Xpert MTB/RIF showed false-positive rifampicin resistance in one-third of cases tested between December 2009 and November 2011 (32). Sequencing of the *rpoB* gene around the mutational hot-spot revealed that the test incorrectly assigned rifampicin resistance to silent mutations in the *rpoB* gene that do not cause any phenotypic change. It was a cautionary finding that underscores the importance of confirmatory testing by sequencing of the *rpoB* gene in rifampicin resistance positive tests before reporting the rifampicin result. This confirmation was required to rule out instances where silent mutations occur. In response to multiple similar reports in the literature about false-positive RIF results, Cepheid has further modified the Xpert MTB/RIF system to overcome this problem and recently introduced the Xpert MTB/RIF Ultra. The newer version uses two multiplex targets, IS6110 and IS1081 to detect the presence of *M. tuberculosis* and rifampicin resistance, especially in resource-constrained countries. More than 95% of RIF resistant mutations are associated with mutations in the *rpoB* gene and the majority of the mutations in *rpoB* gene are found within an 81 base pair (bp) RIF-resistance determining region (RRDR). Hence Xpert MTB/RIF PCR technology integrates this 81bp mutational hot-spot in the *rpoB* gene, and thus not only detects the presence of MTBC DNA in a clinical specimen but also whether there is a RIF resistance-associated mutation in the RRDR hot-spot resulting in presumptive MDR-TB. Mycobacterial resistance to RIF mostly occurs together with resistance to INH and hence resistance to RIF is considered as a surrogate marker for MDR-TB (31).

Another common molecular technology used in gDST for detection of resistance to RIF and other first and second line drugs are the Hain Line probe assays (LPA) - Genotype MTBDRplus and Genotype MTBDRsl. These tests involve a multiplex PCR amplification followed by reverse hybridisation where single-stranded amplicons bind to specific probes attached to the LPA strips. The visualised band patterns on
the strips are then interpreted by a manual comparison with a printed template for the absence/presence of wild-type and mutant bands. Genotype MTBDRplus (both versions 1.0 and 2.0) includes the 81bp mutation hotspot of rpoB for RIF resistance as well as the mutational hotspots in katG and inhA genes for INH resistance. Hain Genotype MTBDRsl version 1.0 detects mutational hotspots in rrs, gyrA and embB genes implicated in the resistance to SLIDs, FQ and ethambutol respectively. The new version 2.0 of MTBDRsl does not carry the embB gene. In its place, the MTBDRsl assay incorporates probes to detect mutations in gyrB and the promoter region of the eis gene, which are associated with resistance to FQs and SLIDs, respectively. (Table 2) (35). Genotypic DST technology is successfully used on AFB smear-positive respiratory specimens as well as MTBC culture isolates (36). The commercial diagnostic tests for pDST and their targets and specific application are tabulated below (table 2).

### Table 2. Commercial molecular diagnostic platforms for gDST and their targets.

<table>
<thead>
<tr>
<th>Type of Therapy</th>
<th>Drug name</th>
<th>Mutation in gene(s) conferring resistance</th>
<th>Commercially available molecular diagnostic platforms for mutation detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>First line</td>
<td>Rifampicin</td>
<td>rpoB</td>
<td>Xpert MTB/RIF (Ultra), Hain Genotype MTBDRplus (V 1.0 and 2.0)</td>
</tr>
<tr>
<td></td>
<td>Isoniazid</td>
<td>katG, inhA</td>
<td>Hain Genotype MTBDRplus (V 1.0 and 2.0)</td>
</tr>
<tr>
<td></td>
<td>Ethambutol</td>
<td>embB</td>
<td>Hain Genotype MTBDRsl (V 1.0 only)</td>
</tr>
<tr>
<td></td>
<td>Pyrazinamide</td>
<td>pncA</td>
<td>No</td>
</tr>
<tr>
<td>Second line</td>
<td>Moxifloxacin/Levofoxacin</td>
<td>gyrA, gyrB</td>
<td>Hain Genotype MTBDRsl/gyrA (V1.0 and V2.0); gyrB in V2.0 only</td>
</tr>
<tr>
<td></td>
<td>Kanamycin/Amikacin</td>
<td>rrs, eis</td>
<td>Hain Genotype MTBDRsl/rrs (V1.0 and V2.0); eis in V2.0 only</td>
</tr>
<tr>
<td></td>
<td>Capreomycin/Viomycin</td>
<td>rrs</td>
<td>MTBDRsl (V1.0 and V2.0)</td>
</tr>
</tbody>
</table>

### TB typing

*M. tuberculosis* has a homogenous genome that harbours little genetic diversity and as such is referred to as monomorphic bacterial pathogen. Most current genotyping techniques used to study the epidemiology of *M. tuberculosis* are based on regions of the genome that have variation (polymorphism) like mobile or repetitive genetic elements (37). TB genotyping, when combined with epidemiological data, has been used as a proxy for TB transmission. The proportion of cases that belong to a genotype cluster are influenced by the discriminatory power of the genotyping approach used. The three most commonly used genotyping methods include (1) insertion sequence 6110 (IS6110) based restriction fragment length polymorphism (RFLP), (2) spacer oligonucleotide typing or spoligotyping and (3) mycobacterial interspersed repetitive units - variable tandem repeat loci (MIRU-VNTR).

In IS6110-RFLP, the mycobacterial genomic DNA is separated by gel electrophoresis. The bands are then transferred to a membrane, and Southern blot hybridization is carried out with a labelled probe complementary to part of the 3’ end of the IS6110 sequence. As a result, every visualized fragment represents a single copy of IS6110 surrounded by different lengths of flanking DNA sequences (38). The IS6110-RFLP method is highly discriminatory and reproducible, and its profiles are stable over time. However, strains with fewer than six IS6110 insertion sites have a limited degree of polymorphism.

Direct repeat (DR) locus is a chromosomal region that contains between 10 to 50 copies of a 36bp direct repeat separated by spacer DNA with various sequences each of which is 37 to 41bp. Spoligotyping detects spacer sequences interspersed with direct repeats (DRs) in the genomic region uniquely present in members of *M. tuberculosis* complex. Strains differ in terms of the presence or absence of specific spacers, the pattern of spacers in a strains can be used for genotyping. Spoligotyping requires a lot less DNA than IS6110-RFLP and can be expressed in a digital format (39).
Variable number of tandem repeats (VNTR) are novel minisatellite-like structure in the genome composed of 40bp to 100bp repetitive sequences which in *M. tuberculosis* is named “mycobacterial interspersed repetitive units” (MIRU). These are scattered in 41 locations throughout the genome of *M. tuberculosis*. MIRU-VNTR based genotyping interrogates multiple genomic loci, mostly in intergenic regions, which contain variable numbers of DNA tandem repeats. Twelve of these 41 locations show polymorphisms in copy number of non-related *M. tuberculosis* isolates. The principle of this typing system is PCR analysis of 12 variable tandem repeat loci with specific primers complementary to the flanking regions followed by gel electrophoresis. The size (in base pair, bp) of the amplicon reflects the tandem repeat unit and is converted into by gel electrophoresis. The size (in base pair, bp) of the scattered in 41 locations throughout the genome of “mycobacterial interspersed repetitive units” (MIRU). These are increasingly also being applied to study evolutionary questions. Epidemiological applications, TB genotyping methods are 100% reproducible, sensitive, and specific for specific primers complementary to the flanking regions followed system is PCR analysis of 12 variable tandem repeat loci with target of susceptibility to first line drugs and *M. tuberculosis*. TB genotyping helps identify TB patients involved in recent transmissions in combination with epidemiological data. It is a method for monitoring epidemiological trends, and has significant impacts on the control of TB. Genotyping could enable the earlier detection of outbreaks, resulting in more rapid responses and fewer transmissions. For the laboratory, genotyping identifies false-positive cultures that may be the result of mislabeling of specimens, or cross-contamination of cultures, and sometimes can address clinical issues where it could differentiate between treatment failure and re-infection in the same patient. Moreover, in addition to routine molecular epidemiological applications, TB genotyping methods are increasingly also being applied to study evolutionary questions.

**Whole genome sequencing and its application in tuberculosis**

Advantage is being taken of the new molecular technology of massively parallel sequencing (MPS) or next generation sequencing (NGS). This technology supersedes the “first generation” Sanger sequencing which targets limited number of DNA sequences at a time. The MPS/NGS technology has been hailed as a highly advanced molecular technology capable of sequencing the entire genome of microbial pathogens including *Mycobacterium tuberculosis*. It provides a lot of information about the genome of the MTBC than is available from the current commercial diagnostic molecular technologies. Whole genome sequencing is now being undertaken on every laboratory confirmed MTBC isolates in the Mycobacterial Reference Laboratories like the Public Health England in order to gather more information from the whole genome of *Mycobacterium tuberculosis* and not just the mutation hotspots involved in resistance to anti-tuberculous drugs. Recently this tool has been used for genotypic prediction of *M. tuberculosis* susceptibility to first line drugs and correlated with phenotypic susceptibility to those drugs (41). Thus it becomes an invaluable technology for gathering information about resistance to multiple drugs used to treat tuberculosis (42). WGS technology is also being used for molecular typing of the *M. tuberculosis* strains to help Public Health in contact tracing (43).

Locally, WGS was used to retrospectively study the first and only XDR *M. tuberculosis* strain in New Zealand. Genomic DST determination based on WGS data was compared to the results obtained from pDST and other commercial gDST technologies previously used to characterise this isolate. WGS provides a higher resolution in gDST of the XDR MTBC isolate (44).

In another instance, WGS of a New Zealand multidrug-resistant *M. tuberculosis* belonging to lineage 3 was compared to that of a drug-susceptible New Zealand MTBC isolate also belonging to lineage 3. Both isolates have identical genotypic profile using the 24-locus MIRU-VNTR. MIRU-VNTR data suggested that these cases were linked epidemiologically. But WGS data analysis showed these two isolates were not genotypically identical and hence not epidemiologically linked. This showed the limitation of MIRU-VNTR typing for *M. tuberculosis* in some cases of infection with specific lineage (like lineage 3) of *M. tuberculosis* (45).

In spite of the low burden of tuberculosis in New Zealand, there is still significant active transmission of tuberculosis. This is especially caused by one specific MIRU-VNTR based genotypic cluster of *M. tuberculosis* complex, informally called the Rangipo (based on the region from which the first laboratory confirmed case was isolated) strain belonging to this cluster. Whole genome sequencing (WGS) was used to investigate the acquired functional advantages of the Rangipo strain over others in their ability to transmit and cause tuberculosis in New Zealand. The data obtained from WGS suggest that strain specific virulence factor variations are important for the successful spreading of the Rangipo strain in New Zealand (46).

![Figure 5. Number of MDR-TB patients in New Zealand confirmed by laboratory from 2002 to 2013](image)

**Number of MDR-TB from 2002-2013**

*Number of patients*

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**New Zealand has a low-burden of MDR-TB** (Figure 5). From 2002 till 2013, there were 38 laboratory confirmed cases of MDR-TB. A retrospective study was undertaken to compare the pDST results of the MDR isolates from 2002 till 2013 with the gDST results obtained from the Cepheid Xpert MTB/RIF and Genotype MTBDRplus. The comparison highlighted discrepancies in the results for resistance to RIF and INH between pDST and gDST in 12/38 (31.5%) MDR-TB isolates (47).

Using WGS technology, additional information was obtained on the drug resistant markers, which addressed the discordance between results from the pDST and the gDST results. In 3/12 MDRs, with discordant result, WGS result showed mutations outside the target *rpoB* gene hotspot for RIF resistance, RRDR which went undetected in the Cepheid Xpert MTB/RIF and Genotype MTBDRplus tests since these technologies look at RRDR regions only.

In the remaining 9/12 MDRs, mutations were either detected only in the *inhA* gene implicated in low level INH resistance though all 9 MDRs showed high INH resistance by pDST (48) or no mutation was detected in *katG* or *inhA* genes using these commercial platforms. Using WGS, additional mutations were
detected in \textit{katG}, and/or \textit{inhA} genes outside the mutation hotspot or altogether in other putative genes implicated in INH resistance.

These results emphasized the utility of WGS in a reference mycobacterium laboratory in a country like New Zealand with a low burden of MDR-TB to supplement the diagnostic molecular tests and to assist in a rapid but accurate diagnosis and appropriate management of MDR-TB and to maintain a low burden (47).

The current bottleneck in application of this WGS technology in routine diagnostic work is analysing the data. WGS data analysis benefits from bioinformatics skills acquired with specialised training. Lack of such training among clinical scientists and microbiologists pose an impediment for its clinical adoption as the introduction of WGS will be hampered by the complexity of data and its analysis. Most clinical laboratories handling such data have developed in-house pipelines for analysis with in-house bioinformatic support. This, in turn, can hinder standardization among laboratories embracing this technology and accreditation like ISO 15189 essential for clinical diagnostic laboratories performing and interpreting WGS (49). Moreover the huge amount of data generated requires robust and secure information technology (IT) infrastructure for transfer and storage of data. Hopefully, with time and with the development of easy-to-use automated analysis pipelines and databases, scientists in clinical laboratories without bioinformatic training will be able to analyse whole genome sequence data in the not-so-distant future.

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