Recent developments in the diagnosis of drug-resistant tuberculosis

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Urgent steps are required to control the drug-resistant tuberculosis (TB) epidemic worldwide. Individualised treatment, using detailed drug-susceptibility test results to guide choice of antibiotics, improves patient outcomes and minimises adverse effects. Recent years have seen substantial advances in our ability to provide rapid, detailed drug-resistance profiles using genotypic methods for detection of mutations conferring drug-resistance. Rapid testing using real-time PCR to target the most important drug-resistance mutations allows the diagnosis of drug resistance to be made with the first diagnostic test, even in low resource settings. The use of whole genome sequencing to infer resistance to a range of different drugs facilitates earlier tailoring of therapy and detection of resistant subpopulations in mixed infections. Low burden countries, such as Australia are well positioned to lead the development and refinement of these new methods, to accelerate the incorporation of these new tools into TB control programs in high burden countries.

While the incidence of new tuberculosis (TB) cases is estimated to be declining at about 2% per year worldwide, with even steeper declines in TB mortality, TB remains the leading infectious cause of death globally[1]. However, sequential surveys of drug-resistance have shown that multidrug-resistant TB (MDR-TB, resistant to the key drugs rifampicin and isoniazid) is not following a similar trend, with countries either reporting increasing rates of MDR-TB, or rates that are declining much more slowly than drug-susceptible disease[1]. In key high-burden countries, such as China, India and South Africa, most new cases of drug-resistant TB arise as a consequence of direct transmission of resistant strains[2], rather than as a result of acquisition of resistance during treatment. In these countries, the drug-resistant TB epidemic is a parallel epidemic, which will not be eradicated even if drug-susceptible TB is controlled.

Treatment of rifampicin-resistant (RR-) or MDR-TB has been lengthy and associated with poor patient outcomes – until recently only about 50% of individuals with MDR-TB have been reported to be successfully treated globally[3]. Encouragingly, treatment outcomes are improving with the use of new and repurposed drugs now available[4]. In well resourced settings, such as Germany, The Netherlands and Canada, individualised treatment, where the regimen is tailored according to the bacterial drug-susceptibility profile, is standard of care. Treatment success levels ≥80% have been reported with individualised treatment, even with regimens not including new or repurposed drugs[5,6]. However, detailed drug susceptibility testing (DST), which is required for individualised treatment, is associated with considerable difficulties, particularly in resource-limited settings, where most individuals with MDR/RR-TB reside. Phenotypic, culture-based DST, which is usually performed using semi-automated liquid culture systems, is slow (≥6 weeks), costly, and poses biosafety risks. Moreover, since DST in liquid culture uses only one or two critical concentrations per drug, it may fail to detect clinically relevant rifampicin resistance[7]. TB isolates exhibit clinically relevant heterogeneity in susceptibility, not captured through culture-based drug-susceptibility testing (DST) using a single drug concentration[8]. Fortunately, there have been substantial advances in recent years in improved diagnostics for drug-resistance and in the resulting ability to tailor treatment based on individual drug-susceptibility profiles. Genotypic drug resistance testing, where resistance is inferred based on the presence of resistance-conferring mutations (Figure 1), is more rapid,

can predict resistance levels, and is now increasingly used in programmatic settings. Since *Mycobacterium tuberculosis* evolves exclusively through chromosomal mutations (horizontal gene transfer is absent), for many drugs there is a clear relationship between specific mutations (usually single nucleotide polymorphisms) and the presence of clinically relevant resistance. The most common resistance-conferring mutations to isoniazid are mutations in *katG* and in the *inhA* promoter, and to rifampicin, mutations in the rifampicin-resistance determining region of the *rpoB* gene. While several 'high confidence' mutations are highly specific for resistance, others are associated with variable, or low-level resistance. Table 1 summarises the key advantages and disadvantages of different methods for detection of drug-resistance in *M. tuberculosis*.

Rapid targeted genotypic identification of drug-resistance

The implementation of the Xpert MTB/RIF test (Xpert), a semi-automated, cartridge-based molecular diagnostic has revolutionised TB diagnosis[9]. Since Xpert uses probes targeting the *rpoB* gene it is able to rapidly identify the presence of TB as well as resistance to rifampicin in the majority of patients with TB. The test is not perfect; sensitivity for TB diagnosis (the proportion of patients with TB who have a positive test) is sub-optimal in patients with HIV infection, false-positive rifampicin resistance calls occur (reduced specificity)[10] and geographically localised strains of TB with mutations outside of the rifampicin-resistance determining region give rise to false susceptible results[11]. Xpert is not able to identify resistance to isoniazid, and has a turnaround time of approximately two hours – too long for a true point of care test. However, Xpert has enabled near universal screening for rifampicin resistance in many settings where drug-resistance testing was not previously available, and sensitivity and specificity for rifampicin resistance are high (95% and 99%, respectively)[12]. A newer version of the test, Xpert MTB/RIF Ultra, has higher sensitivity for TB detection, and uses a different strategy for identification of rifampicin resistance (melt-curve analysis)[13], which, in theory, should be more accurate, although this has not yet been confirmed in clinical studies.

Alternative, or complementary, rapid genotypic tests for resistance have also been introduced. Line probe assays, such as the Genotype MTBDR*plus* test, which are based on reverse hybridisation of PCR amplicons to probes immobilised on strips allow rapid detection of resistance to both isoniazid (*katG* and *inhA* promoter targets) and rifampicin[14]. Although these tests can be used for direct testing of patient samples[15], the tests are technically more complex and, since they require open hybridisation steps, carry the risk of amplicon contamination. They are therefore more typically used for rapid genotypic detection of resistance-conferring mutations in cultured isolates of *M. tuberculosis*. The Genotype MTBDR*sl* test, which is able to detect mutations in the *gyrA* and *gyrB* genes (conferring resistance to fluoroquinolones) and the *rrs* gene and *eis* promoter (conferring resistance to the injectable drugs kanamycin, amikacin

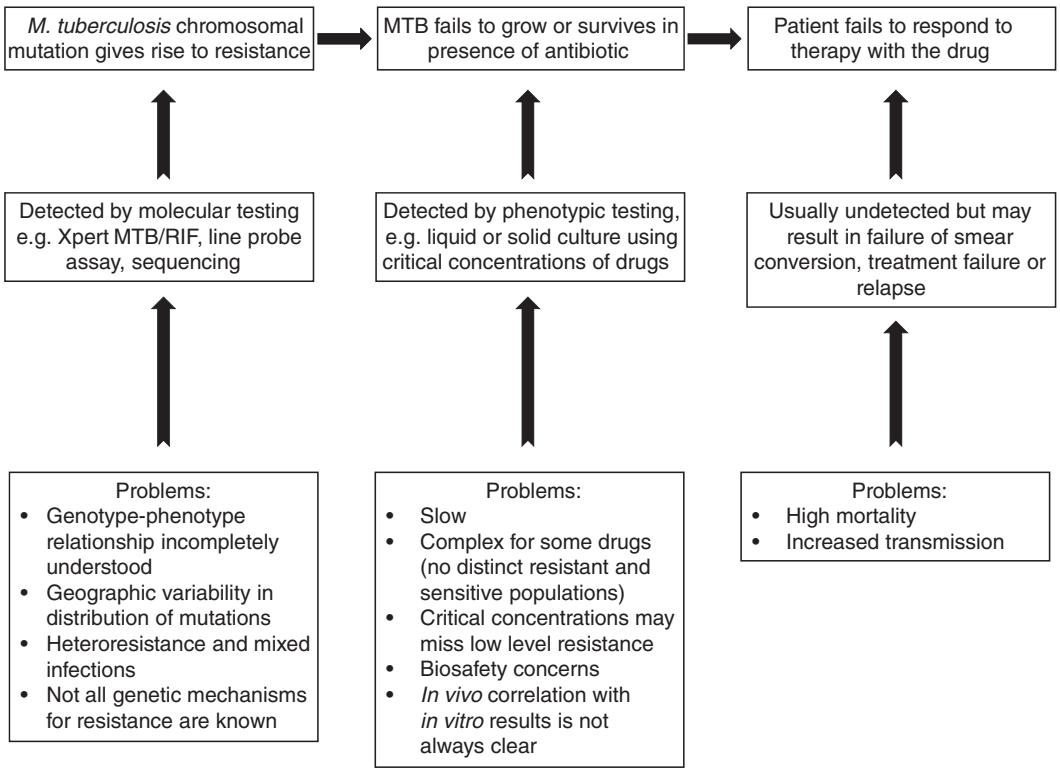

Figure 1. Approaches to detection of drug resistance in *Mycobacterium tuberculosis*.

Table 1. Advantages and disadvantages of commonly used methods for drug susceptibility testing of *Mycobacterium tuberculosis*.

		Examples	Principle	Advantages	Disadvantages
Phenotypic drug susceptibility testing		Liquid media (Mycobacterial Growth Indicator Tube) or solid media	Relative growth of *M. tuberculosis* in the presence or absence of drug	• Direct assessment of growth in presence of the drug • Reference standard method	• Slow, biosafety concerns, technically complex • Use of single critical concentration may miss clinically relevant resistance and mixed infections
Genotypic methods	Line probe assays	Genotype MTBDR*plus* and MTBDR*sl*	PCR amplification of target genes and detection with probes hybridised to membrane	• Accurate • Able to identify the most common mutations and some mixed infections	• Variable performance with direct testing of specimens with low bacillary load • Prone to cross-contamination by amplicons • Technically complex
	Real-time PCR	Xpert MTB/RIF (and Xpert Ultra)	Cartridge-based automated extraction and real-time PCR	• Simple and rapid • Direct testing of sputum • Sensitive	• Only tests for rifampicin resistance • Does not identify which mutation is present
	Whole genome sequencing	MiSeq (Illumina) MinION (Nanopore)	Sequencing followed by standardised analysis pipeline	• Able to detect all resistance-conferring mutations • May detect mixed infections	• Requires cultured isolate • Relatively costly • Requires expertise

and capreomycin) is particularly useful for rapidly testing specimens from patients who have an Xpert test showing resistance to rifampicin, to rule out extensive drug resistance (XDR-TB, MDR-TB with additional resistance to a fluoroquinolone and injectable drug)[16].

Several new rapid molecular diagnostics are coming to market. The BD MAX MDR-TB assay, from Becton Dickinson, is a real-time PCR test that detects resistance to rifampicin (*rpoB*) and isoniazid (*inhA* and *katG*) using raw sputum or sputum sediment. It allows batch processing of 24 samples in four hours and is suitable for centralised laboratory testing[17]. Results from a multicenter diagnostic accuracy study are expected shortly. An Xpert MTB/XDR cartridge that targets *katG* and the *inhA* promoter for isoniazid, *gyrA* for the fluoroquinolones, and *rrs* for kanamycin and amikacin was also recently evaluated[18].

In general, since they target the same genomic regions, these rapid PCR-based molecular diagnostics show similar performance characteristics for detection of resistance. Sensitivity for rifampicin resistance is typically above 95%, for isoniazid around 85%, for fluoroquinolones approximately 90% and for injectable drugs 70%. Specificity is usually high (approximately 95%) for all of these drugs. Choice of an assay may therefore depend on other features, such as sensitivity for TB detection, whether one is testing a sputum sample or a cultured isolate, laboratory infrastructure, workload and cost.

PCR tests for drug-resistance are therefore accurate and useful for direct testing of samples where low numbers of bacilli are present; however, they are limited by the number of targets that may be amplified and detected in a single test, and so are not able to provide detailed susceptibility profiles. Indeed, given that the World Health Organization have recently changed treatment guidance for MDR/RR-TB to no longer recommend injectable drugs for the majority[19], the relevance of tests targeting *rrs* for injectable drug resistance is now limited.

Whole genome sequence-based prediction of resistance

Whole genome sequencing (WGS) of *M tuberculosis*, where all potential resistance-conferring mutations are identified simultaneously, is increasingly being used to predict detailed drug susceptibility to individualise RR-TB treatment[20]. A key challenge is the limitation in current understanding of genotype-phenotype associations, particularly for second-line and new/repurposed drugs, such as bedaquiline, delamanid and linezolid. To address this, two international collaborations (ReSeqTB[21], CRyPTIC[22]) are compiling

WGS data and matched phenotypic DST from different settings. A recent study of phenotype-genotype correlation amongst 10,209 isolates showed that genotype correctly predicted phenotypic resistance to isoniazid, rifampicin, ethambutol, and pyrazinamide with 97.1%, 97.5%, 94.6%, and 91.3% sensitivity, and 99.0%, 98.8%, 93.6%, and 96.8% specificity respectively[22].

WGS-based prediction of resistance, using DNA extracted from cultured isolates, has been implemented programmatically in several settings including in the United Kingdom[23,24] and in parts of Australia[25]. WGS is cost-competitive, when considered with a comparator of detailed phenotypic DST plus strain genotyping (to allow molecular epidemiological investigation of linked cases), and more rapid[23]. Pipelines for analysis are still not fully standardised[26], and several bioinformatics tools exist (TBProfiler, MyKrobe, KvarQ, PhyResSE)[27]. There is also no consensus on criteria for detection of mixed infections (with mixed populations of susceptible and resistant bacilli), and the related issue of the sequence coverage needed to detect resistant sub-populations.

At present a major limitation of WGS is that cultured isolates are required to provide sufficient pure *M. tuberculosis* DNA for sequencing. Growing evidence suggests that culture masks clinically relevant heterogeneity in drug resistance[28]. Methods for direct sequencing of *M. tuberculosis* DNA in patient specimens are being developed[29], but these are currently insensitive and complex.

Finally, analytical and bioinformatics platforms for whole genome sequencing are best suited to large centralised laboratories, and so their applicability in low resource settings, or closer to the point of care is unclear. Several groups have explored the use of Nanopore sequencing of *M. tuberculosis* isolates[30], which may be better suited to low volume, low-resource settings, with some success.

Tackling the drug-resistant TB epidemic will require rapid diagnosis and effective treatment of a large proportion of the estimated global burden of disease in order to interrupt transmission. WGS for drug resistance prediction, combined with the availability of new drugs, offers the possibility of improved patient outcomes through individualised treatment. Realising this goal in resource-limited settings, while difficult, will ensure that all individuals with DR-TB have access to the same standard of care[31].

Conflicts of interest

The authors declare no conflicts of interest.

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