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REVIEW ARTICLE

Clinical implications of molecular drug resistance testing for *Mycobacterium tuberculosis*: a TBNET/RESIST-TB consensus statement

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SUMMARY

The emergence of drug-resistant strains of *Mycobacterium tuberculosis* is a challenge to global tuberculosis (TB) control. Although culture-based methods have been regarded as the gold standard for drug susceptibility testing (DST), molecular methods provide rapid information on mutations in the *M. tuberculosis* genome associated with resistance to anti-tuberculosis drugs. We ascertained consensus on the use of the results of molecular DST for clinical treatment decisions in TB patients. This document has been developed by TBNET and RESIST-TB groups to reach a consensus about reporting standards in the clinical use of molecular DST results. Review of the available literature and the search for evidence included hand-searching journals and searching electronic databases. The panel identified single nucleotide mutations in genomic regions of *M. tuberculosis* coding for *kat*G, *inh*A, *rpo*B, *emb*B, *rrs*, *rps*L and *gyr*A that are likely related to drug resistance in vivo. Identification of any of these mutations in clinical isolates of *M. tuberculosis* has implications for the management of TB patients, pending the results of in vitro DST. However, false-positive and false-negative results in detecting resistance-associated mutations in drugs for which there is poor or unproven correlation between phenotypic and clinical drug resistance complicate the interpretation. Reports of molecular DST results should therefore include specific information on the mutations identified and provide guidance for clinicians on interpretation and on the choice of the appropriate initial drug regimen.

KEY WORDS: clinician guidance; interpretation; molecular methods

WHILE THE GLOBAL INCIDENCE of tuberculosis (TB) has declined in recent years, the emergence of drug-resistant strains of *Mycobacterium tuberculosis* is a challenge to TB control in many parts of the world.[1] Treatment for *M. tuberculosis* has been available for over 60 years. During this time, we have observed the emergence of multidrug-resistant TB (MDR-TB), which is formally defined as resistance to at least isoniazid (INH, H) and rifampicin (RMP, R). We have also observed the development of extensively drug-resistant TB (XDR-TB), defined as MDR-TB with additional resistance to any fluoroquinolone (FQ) and one of the second-line injectable drugs, kanamycin (KM), amikacin (AMK) or capreomycin (CPM), and, most recently, the development of so-called totally drug-resistant strains.[2,3] According to the World Health Organization (WHO), 136 412 patients were notified with MDR-TB in 2013 worldwide. The average proportion of MDR-TB cases with XDR-TB was 9.0%.[1] Estimated

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numbers of patients with MDR-TB are 3.5 times higher, at approximately 480 000 (credibility range 350 000–610 000).[1]

Effective TB control depends upon rapid case detection and initiation of adequate treatment. Conventional procedures for the isolation of *M. tuberculosis* and DST are slow, causing substantial delays until patients with drug-resistant TB receive adequate treatment. Important aspects regarding the molecular basis of anti-tuberculosis drug resistance have recently been elucidated. Molecular methods based on genomic DNA sequencing have been used to detect the main mutations involved in drug resistance. New molecular methods, such as polymerase chain reaction (PCR) based solid-phase reverse hybridisation line-probe assays (LPAs), have been developed to detect the most common mutations conferring *M. tuberculosis* drug resistance.[4] In addition, technologies for sequencing and analysing the whole genome of *M. tuberculosis* have become available to guide physicians on the treatment selection for patients with drug-resistant TB.[5] These methods are sensitive and specific when detecting resistance mutations in bacterial isolates and also in clinical samples.[6,7]

Although the WHO recommends LPAs for rapid molecular diagnosis of RMP and INH resistance, these tests are not currently recommended by the WHO for rapid second-line DST in *M. tuberculosis*. A recent Cochrane review found that in adults with TB,[8] a positive LPA result (GenoType® MTBDR*sl*, Hain Lifescience, Nehren, Germany) for FQ resistance, second-line injectable drug resistance or XDR-TB can be treated with confidence. However, current generations of LPAs cannot detect approximately one in four cases of second-line injectable resistant TB, and will miss between one in four and one in three cases of XDR-TB. However, despite the absence of an official WHO recommendation, LPAs are frequently used in clinical practice for the initial diagnosis of second-line drug resistance patterns of *M. tuberculosis* in patients with MDR-/XDR-TB. In very few specialised centres, whole genome sequencing (WGS) is already implemented for molecular DST in TB.[9]

To guide clinicians in the initial treatment of MDR-/XDR-TB patients, we summarise current knowledge on the ability of molecular methods to predict in vitro drug resistance of first- and second-line anti-tuberculosis drugs, and present a minimal consensus on which information obtained from molecular DST should influence initial treatment decisions in such patients.

METHODOLOGY

This document has been developed by physicians, microbiologists, molecular biologists and clinical epidemiologists of the TBNET (www.tb-net.org) and RESIST-TB (www.resisttb-org) groups to reach a consensus about reporting standards in the clinical use of *M. tuberculosis* molecular DST results. Review of the available literature and the search for evidence included hand-searching journals and searching electronic databases including MEDLINE and PubMed. Consensus statements were developed in a stepwise approach:[10]

Step 1: Preliminary proposals for key recommendations were drafted by the coordinating author (JD). All co-authors were asked to provide alternative statements.
Step 2: Alternative statements were collected from co-authors.
Step 3: Co-authors were asked to select one preferred statement among the alternative statements. The co-authors were blinded to the vote.
Step 4: For each recommendation, the statement that received most votes was selected for inclusion in the manuscript.
Step 5: All co-authors were asked to indicate their agreement, disagreement or whether they preferred to abstain from a decision.

TREATMENT OF TUBERCULOSIS PATIENTS

Anti-tuberculosis drugs are categorised by the WHO in groups, from the most effective, most commonly used drugs (Group 1) to those that are rarely used and have unclear effectiveness (Group 5). These drugs work through a variety of mechanisms, as outlined in the Appendix (Appendix Table A.1).* More detailed information on these mechanisms can be found in several excellent recent reviews.[11]

Recommendations for treatment regimens for drug-susceptible TB were developed following a series of clinical trials over a 20-year period, culminating in the currently recommended 'standard regimen' consisting of INH, RMP, pyrazinamide (PZA, Z) and ethambutol (EMB, E) (HRZE) for 2 months, followed by INH and RMP for 4 months.[12] The WHO currently recommends the following strategy:[13]

1 In the treatment of MDR-TB patients, an FQ should be used (strong recommendation, very low-quality evidence).
2 In the treatment of MDR-TB patients, a later-generation FQ rather than an earlier-generation FQ should be used (conditional recommendation, very low-quality evidence).

*The appendix is available in the online version of this article, at http://www.ingentaconnect.com/content/iuatld/ijtld/2015/00000020/00000001/art00007

3 In the treatment of MDR-TB patients, ethionamide (ETH) (or prothionamide [PTH]) should be used (strong recommendation, very low-quality evidence).
4 In the treatment of MDR-TB patients, four second-line anti-tuberculous drugs that are likely to be effective (including a parenteral agent), as well as PZA, should be included in the intensive phase (conditional recommendation, very low-quality evidence).
5 In the treatment of MDR-TB patients, regimens should include at least PZA, a FQ, a parenteral agent, ETH (or PTH), and either cycloserine (CS) or para-aminosalicylic acid (PAS) if CS cannot be used (conditional recommendation, very low-quality evidence).

The abovementioned WHO recommendations result largely from the paucity of drugs that are effective and well tolerated, and from the fact that many patients with drug-resistant disease have isolates that are resistant to considerably more drugs than merely INH and RMP. These regimens have substantial toxicity,[14–18] and effectiveness was estimated at 54–66% in two meta-analyses.[19,20] Programmatic data suggest that fewer than 50% of patients successfully complete treatment.[1]

Ideally, treatment regimens should be based on in vitro DST of the patient's *M. tuberculosis* isolates, but phenotypic testing can take 6–8 weeks. In addition, in many settings phenotypic testing of first- and/or second-line drugs is not possible and empirical treatment is prescribed. Many patients are therefore treated with suboptimal regimens for prolonged periods. The advent of DNA-based diagnostics offers the potential for rapid assessment of susceptibility and prompt administration of the optimal regimen, within the context of current guidelines.[10]

In the past few years, several new agents have been developed that offer new hope for improved MDR-TB treatment regimens. These agents comprise either new drug classes (diarylquinolines, nitroimidazole derivatives) or new agents in classes in which current agents are relatively toxic (oxazolidinones).[21] Two new drugs, bedaquiline, the first approved agent in the diarlyquinolone class, and delamanid, the first approved agent in the nitroimidazole derivative class, have recently become available for clinical use. Clinical trials are urgently needed to determine which companion drugs will lead to the best clinical outcomes for MDR-TB patients. Until recently, there were limited possibilities of determining if a patient's isolate was susceptible to these agents, because neither bedaquiline nor dalamanid were available from the manufacturer for laboratory in vitro DST. In addition, standardised methodologies suitable for widespread adoption by reference laboratories have not been fully developed or established. [22,23]

In conclusion

1 Until recently, there were no systematic trials of regimens for the treatment of drug-resistant TB.
2 Most experts think that regimens should be designed to include at least four drugs to which isolates are susceptible in vitro.
3 Phenotypic testing takes 6–8 weeks, resulting in substantial delays in optimising MDR-TB treatment regimens.
4 Phenotypic DST against second-line drugs is not available in many areas.

EVOLUTION OF DRUG RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

M. tuberculosis complex develops drug resistance as a result of spontaneous mutations in genes encoded on the chromosome. These mutations include single nucleotide changes, small insertions and deletions (*indels*) or larger deletions, and either modify the drug target itself, silence drug activating enzymes in the case of pro-drugs, or circumvent drug action by increasing the gene product targeted by the drug.[11] The bacterial cells carrying such mutations are selected during periods of ineffective patient treatment and increase in frequency, eventually replacing the drug-susceptible bacterial population.[24] The probability of acquiring resistance through spontaneous mutations varies by drug, ranging from approximately 1 in 10^8 bacilli for RMP, to approximately 1 in 10^6 bacilli for INH, streptomycin (SM) and EMB.[25] Moreover, *M. tuberculosis* comprises various phylogenetically distinct lineages,[26] and recent studies indicate that the rate of mutation towards drug resistance might be influenced by the lineage to which a particular strain belongs.[27,28]

Drug-resistant subpopulations of bacteria may be selected in patients treated with only one effective drug.[29–31] The main reasons for phases of monotherapy include improper prescription of treatment regimens, addition of single drugs to failing treatment regimens, inadequate drug supply, patient non-adherence, quality of the anti-tuberculosis drugs (a very relevant reason in many settings), differences in pharmacogenomics, and the pharmacodynamic and kinetic properties of the drugs administered.[32] The development of multidrug resistance results from several periods of 'sequential monotherapy' during which resistance to other drugs is acquired, a phenomenon referred to as amplification of drug resistance. Recent data indicate that *M. tuberculosis* strains evolve within individual patients during treatment, and that this micro-evolution is dynamic, leading to the presence of different subpopulations of bacteria with divergent sets of drug resistance mutations.[29,33] This intra-patient diversity is likely

to influence the performance of molecular and phenotypic DST, and needs to be considered when interpreting routine diagnostic results.

An important factor driving the current MDR-TB epidemic is the direct transmission of MDR-TB strains, leading to high proportions of MDR-TB strains in patients who have not been treated previously.[34] Because of the frequent delays in diagnosing MDR-TB, strains from these patients with primary MDR-TB are at a high risk for developing further drug resistance.[35,36] Undetected MDR-TB and the less effective second-line treatment regimens also contribute to prolonged periods of sputum smear positivity among MDR-TB patients, enhancing the role of transmission of MDR-TB strains.[32,35] The overall impact of transmission as opposed to de novo acquisition of drug resistance on the global MDR-TB epidemic has been a subject of controversy for a long time due to a postulated lower fitness of MDR-TB strains of *M. tuberculosis*.[37,38] Early data posited that the genetic mutations conferring drug resistance in *M. tuberculosis* resulted in reduced bacterial fitness,[39] leading to the assumption that drug-resistant strains would not disseminate widely in the community.[37,40] However, it has become clear that resistance mutations with no or low fitness impact exist that facilitate the spread and amplification of resistance.[41–43] In addition, more recent studies have indicated that some drug-resistant *M. tuberculosis* strains have acquired compensatory mechanisms that restore the fitness cost associated with resistance mutations,[44–46] leading to the expansion of particular highly transmissible MDR-TB clones in different areas of the world.[47] The interaction between a specific drug resistance-conferring mutation and a compensatory mutation is an example of epistasis, which occurs when the phenotypic effect of one mutation is modified by the presence of a second mutation.[48]

In conclusion

1 Large studies are necessary to establish the relationship between the mutations detected and the phenotype finally expressed by *M. tuberculosis*.
2 The correlation with clinical outcome has not been investigated.
3 An international database with validated drug resistance mutations should be established.
4 Clinical trials are necessary to demonstrate the usefulness of individualised treatment regimens based on multi-analyte molecular assays.

PRINCIPLES OF PHENOTYPIC DRUG SUSCEPTIBILITY TESTING

Phenotypic DST of mycobacteria assesses the ability of the organism to grow in the presence of the antibiotic using either solid or liquid medium. Various methods have been described (absolute concentration, resistance ratio, proportion methods, in a variety of commercial and non-commercial systems),[49–53] but only the commonly used proportion method will be outlined, as it is often used as a reference standard.

The proportion method is based on the premise that if <1% of the organisms in a given population are resistant to a drug at a given concentration (the so-called critical concentration), the population as a whole is susceptible, and conversely, if >1% of the organisms are resistant, the population as a whole is resistant. The critical concentration represents the lowest concentration of the agent that inhibits >95% of wild-type (wt) (susceptible) strains.[54] Thus, the critical concentration basically corresponds to what is known as epidemiological cut-off (ECOFF).[55] The organism is inoculated onto drug-free and drug-containing medium, and following incubation, the number of colonies is compared to calculate the proportion of resistant colonies. When performed in liquid culture, growth in an antibiotic-containing medium (using a critical concentration) is compared to growth in an antibiotic-free medium. If the drug-free medium registers growth before the drug-containing medium, the isolate is regarded as susceptible, and vice versa for the determination of resistance. In many commercial systems, the inoculum in the antibiotic-free medium is a 1:100 dilution of the inoculum in the antibiotic-containing medium reflecting the proportion method. It is important to note that resistance as defined here is a technical term: it does not correspond to clinical resistance and it is not to be confused with mutational resistance, the driver of acquired drug resistance in *M. tuberculosis*.[56] In combination with the critical concentration, the critical proportion is a laboratory term used in in vitro DST to define the epidemiological cut-off.

The critical concentrations of many antibiotics were published by the WHO in 2008, with updates suggested at a meeting in 2012 (these updates have not yet been formally published by the WHO) (Appendix Table A.2).[57,58] Results using current critical concentrations are generally accurate and reproducible for RMP and INH, but less so for EMB and SM.[59,60] Although critical concentrations have been recommended for PZA testing, phenotypic testing for this drug is technically difficult, given the drug's activity at a low pH, which inhibits mycobacterial growth.[61] Concerns have also been raised about the appropriateness of the critical concentration for PZA as well as the reliability of the current methods compared to molecular detection of resistance mutations.[62,63] Regarding second-line agents, the critical concentrations for FQs and injectable agents (AMK, KM and CPM) are currently appropriate,[60,64,65]

although the evidence is not as strong as for RMP and INH.

A drawback to the use of critical concentrations is that it assumes that there are two clearly defined populations of organisms (resistant and susceptible), and that the minimum inhibitory concentration (MIC) distributions for these two populations can be easily separated. If the MICs of resistant and susceptible bacilli are close together or form more of an MIC continuum, then the use of critical concentration to separate resistant from susceptible is problematic[66] and may lead to the variability in results discussed above. The relative proportions of strains with different MICs circulating in the community will also affect the accuracy of phenotypic DST. For example, if there is a relatively high prevalence of strains with low-level resistance, phenotypic testing may not correlate as well with the clinical outcome as it would when strains with 'high-level' resistance are more common and the current critical concentrations would separate susceptible strains more easily from resistant strains.[67] The other problem associated with the use of critical concentrations is that, in some instances, the critical concentration is close to the serum levels attained using standard dosing regimens; this is especially true for second-line agents.[66,68]

The use of two critical concentrations (low and high) has been advocated when testing certain antibiotics[54,57] such as INH, EMB and SM. Resistance at the high concentration indicates resistance, while an isolate resistant at the low concentration, but susceptible at the high concentration, suggests low-level resistance, and higher doses of the respective drug may still be clinically effective. However, there is a need to critically re-evaluate many of the current critical concentrations as well as testing methodologies to better standardise phenotypic resistance testing for *M. tuberculosis*, and to better understand the correlation between the phenotypic DST result and treatment outcomes. It is most likely that quantitative measures for drug susceptibility need to be implemented in diagnostic mycobacteriology.[66,69–71]

In conclusion

1 While phenotypic DST is still commonly regarded as the gold standard for determining the susceptibility of *M. tuberculosis* to various drugs, it has a number of limitations.
2 The DST results for some drugs (such as INH and RMP) are more reliable than for other drugs (such as EMB).
3 There is a lack of good clinical outcome data to correlate with the phenotypic DST results for some agents.
4 A good understanding of local epidemiology and molecular resistance mechanisms is important to appropriately interpret phenotypic DST results.

PRINCIPLE OF GENOTYPIC DRUG SUSCEPTIBILITY TESTING

Drug resistance in *M. tuberculosis* is mainly due to single nucleotide mutations (SNMs) that accumulate over time on specific genes. For some antibiotics, the association between the mechanisms of resistance and the responsible genes are very well known, whereas for others we still have incomplete knowledge. Not all of the SNMs detectable in strains showing a resistant phenotype are responsible for drug resistance: some are phylogenetic markers or cause silent mutations.[66,69,70] The frequency of the mutations is also different for the different genes associated with drug resistance.

Molecular detection of the SNMs associated with drug resistance is the fastest way to design a personalised treatment regimen, and it also has the potential to become a bedside technology. WHO-endorsed commercial methods for drug resistance detection include LPAs and the Xpert® MTB/RIF assay (Cepheid, Sunnyvale, CA, USA).[72,73] The specificity and sensitivity of these tests have been evaluated against liquid culture and phenotypic DST as gold standard.[74] The use of 'phenotypic DST' as gold standard for the evaluation of molecular tests was recently challenged.[75] In the future, the use of multiple standards based on sequencing, quantitative DST and clinical outcomes should be considered.

Current molecular techniques detect both live and dead bacteria, and a positive result does not imply the viability of the pathogen. These methods cannot therefore be used for monitoring treatment response.[76]

Line-probe assay

Commercial systems based on DNA probe assays are available for the detection of the most frequent mutations responsible for resistance to RMP, INH, FQs, second-line injectable drugs and EMB.[77–83] The regions of interest are investigated with wt probes, which, in the presence of mutations, fail to hybridise. Most of the systems include confirmatory probes designed to detect the more frequent mutations.

Different commercial assays detect RMP resistance by targeting the hot spot of the *rpo*B gene, known as the RMP resistance-determining region, which harbours more than 95% of mutations responsible for RMP resistance.[84,85] This region is covered in the available LPAs by a number of overlapping wt probes. Five probes are present in INNO LiPA Rif.TB® (Fujirebio, Ghent, Belgium), eight in GenoType® MTBDR*plus* (Hain) and three in the AID TB Resistance assay (Autoimmun Diagnostika GMBH,

Strassberg, Germany) to detect possible mutations in almost the same *rpo*B region using missed hybridisation. INNO LiPA[86] and GenoType MTBDR*plus*[87] include four mutated probes specific to the mutations Asp→Val at codon 516, Ser→Leu at codon 531, and His→Tyr or His→Asp at codon 526 (Appendix Table A.3). The AID TB Resistance assay includes a total of three probes to detect mutations Asp→Val or Asp→Tyr at codon 516, Ser→Leu or Ser→Trp at codon 531, and His→Tyr, His→Asp or His→Arg at codon 526.[88]

Mutations confirmed by mutated probes are most frequently detected in RMP-resistant strains. Mutations at codons 516, 526 and 531, other than those recognised by mutated probes, may also be associated with high-level RMP resistance. Certain mutations have been reported not to be detected by phenotypic susceptibility testing,[89–91] particularly codons that are prone to silent mutations and do not affect drug susceptibility (Appendix Table A.3).

GenoType MTBDR*plus* includes the detection of mutations responsible for INH resistance. In the *kat*G gene, the codon 315 is investigated with a wt probe and two mutated probes specific for the ACC or ACA mutations, both of which are responsible for the change Ser→Thr. In the promoter region of the *inh*A gene, three positions are monitored: the upstream position –16 is targeted by a wt and a mutated probe aiming to recognise the A to G mutation; in the upstream –15 position, a wt and a mutated probe can identify the C to T mutation; while in the upstream position –8, a wt and two mutated probes can discriminate between the T to C or T to A mutations. The AID TB Resistance assay includes two wt probes targeting the *inh*A (positions –16, –15 and –8) and *kat*G codon 315, respectively, and two mutated probes that detect mutations –16G, –15T, –8A and –8C in *inh*A, and S315T in *kat*G, respectively.

Mutations at codon 315 are detected in 60–80% of high-level INH-resistant strains.[92] Mutations in the *inh*A gene promotor are present in 10–20% of resistant strains and are frequently responsible for low-level resistance. The *M. tuberculosis* lineage can influence the level of INH resistance conferred by *inh*A or *kat*G mutations.[93] These mutations also affect susceptibility to ETH.

FQ resistance is investigated by GenoType MTBDR*sl* using a set of three wt probes covering codons 85–96 of the *gyr*A gene and by six mutated probes specific for mutations at codon 90, 91 and (four probes) 94. The AID TB Resistance assay includes one wt probe and six probes to detect mutations in codons 90, 91 and 94 of *gyr*A. Mutations in the *gyr*A gene are present in about 70% of the strains resistant to FQs,[6] although they may not be as useful for reflecting resistance to the later generations of FQs, such as moxifloxacin (MFX).

The detection of resistance to second-line injectable drugs in GenoType MTBDR*sl* is focused on two specific mutations, A1401G and A1484T, in the *rrs* gene. For this purpose, two wt and two mutated probes are used. In the AID TB Resistance test, two wt probes target *rrs* positions 1401/1402 and 1484, respectively, while there are three mutated probes to detect changes in A1401G, C1402T and G1484C/T. According to the review published by Georghiou et al., most of the strains with A1401G, C1402T or G1484T mutations were resistant to second-line injectable drugs. It is of note that 7% of CPM-susceptible isolates carried the A1401G mutation.[94]

SM resistance is only assessed by the AID TB Resistance assay. This test includes a total of three wt probes covering codons 43 and 88 of the *rps*L gene, and *rrs* positions 513 to 517. Most of the strains harbouring the targeted mutations in *rps*L and *rrs* are resistant to SM.[95–97] Seven mutations are targeted by the assay: *rps*L K43R, K88R and K88Q, and *rrs* C513T, A514C, G515C and C517T. Resistance to EMB is investigated at the level of codon 306 of the *emb*B gene with a wt and two mutated probes discriminating the mutations Met→Ile and Met→Val. The AID TB Resistance test includes one wt probe and four mutation probes: M306V, M306I ATA, M306I ATC and M306I ATT. Mutations at codon 306 are present in about 55% of EMB-resistant strains.[6] The clinical significance of strains with a mutation in the presence of a susceptible phenotypic result is as yet unclear.[98,99]

A second generation GenoType MTBDR*sl* is currently under evaluation: the main differences consist in the addition of the mutation in the *eis* gene associated with resistance to KM and in the absence of codon 306 of *emb*B.

A new LPA that has recently been commercialised (Nipro Corporation, Osaka, Japan) is designed to detect *pnc*A mutations associated with PZA resistance. In the only publication available to date,[100] the agreement between LPA and the phenotypic method was low. As the MICs of these PZase-positive PZA-resistant isolates with wt *pnc*A were very low using the BACTEC™ 460™ (BD, Sparks, MD, USA) method, they may have shown false resistance due to the acidity of the medium used for PZA DST, which inhibited *M. tuberculosis* growth.

Xpert MTB/RIF

The Xpert assay is an integrated micro-fluidic based system comprising a GeneXpert instrument and Xpert test cartridges. The system uses an automated protocol with simultaneous DNA amplification, and is based on molecular beacons technology in which each probe is labelled with a different fluorescent dye, permitting simultaneous detection with in-built controls. The PCR target for RMP resistance is the 81 bp region of the *rpo*B gene. The assay flags the presence

of resistance in the absence of binding of wt probes to the target sequence. The pooled sensitivity and specificity of Xpert for RMP detection are respectively 95% (95% confidence interval [CI] 90–97) and 98% (95%CI 97–99). The test is recommended by the WHO as the initial diagnostic test for adults and children presumed to have MDR-TB or human immunodeficiency virus (HIV) associated TB.[101]

Multiplex polymerase chain reaction

Another approach for the detection of mutations is multiplex PCR. Several in-house assays have been developed to detect resistance to first-line and, to a lesser extent, second-line drugs. Depending on the assay, the presence of mutations associated with drug resistance is detected by the presence or absence of an amplification curve. To our knowledge, only one multiplex PCR test is commercially available, the Anyplex™ II MTB/DR/XDR detection kit (Seegene, Seoul, Korea), but only one study has been published.[102] An important drawback of this method is that it is not possible to identify the specific mutation involved.

Platforms for simultaneous detection of multiple mutations

Knowledge about mutations associated with a resistant phenotype is increasing, with the wide accessibility of Next Generation Sequencing (NGS) technology that allows the collection of a large amount of data in a short time at a relatively low cost. Most studies perform WGS from cultured isolates.[9,103] Brown et al. reported the successful and accurate sequencing of *M. tuberculosis* genomes directly from uncultured sputum samples.[104] This alternative could further reduce delays, allowing more personalised treatment.

Microarray-based platforms will allow the comprehensive detection of resistance mutations for first- and second-line drugs, overcoming the limitations of current rapid molecular tests, which can only analyse a few genetic targets. Similar to LPAs, but on a larger scale, these platforms will allow both the detection of the wt sequence and the identification of the specific substitution.

NGS can be considered as the approach of the future for drug resistance detection. Several NGS platforms with different technical characteristics and throughput are available. Different approaches may be considered, from multiplexing several target genes to full genome sequencing. Because of the depth of the information obtained, raw sequence data will be of little use without a highly developed user-friendly software package to interpret results, and it will take enormous efforts to correlate the genomic findings with clinical data. The lack of clinical correlation is a huge problem, and much more so for genomic sequencing data than for phenotypic results.

In conclusion

1 Molecular tests targeting mutations associated with drug resistance have high specificity and sensitivity when compared to DNA sequencing as gold standard.
2 Multiple molecular platforms with different levels of automation are available (and more will be in the future) for the detection of mutations in *M. tuberculosis*.
3 Uncertainty about the correlation between single nucleotide polymorphisms and phenotypic DST, and lack of data correlating mutations to clinical outcomes, is delaying our capacity to use genotypic results to guide personalised patient management.
4 Only full genome sequencing on an extremely large number of strains collected worldwide, coupled with phenotypic DST results, drug treatment and clinical outcomes data, can provide the appropriate statistical power to identify the subset of mutations predictive of treatment failure to any given drug.

RELATIONSHIP BETWEEN THE RESULTS OF GENOTYPIC AND PHENOTYPIC DRUG SUSCEPTIBILITY TESTING AND CLINICAL OUTCOME

Molecular tests may show discordant results when compared to phenotypic DST based on critical concentration testing.[74,98,105–112] The clinical consequences of the limitations in the accuracy of molecular resistance assays depend on the drug, but follow a general pattern. False-positive test results will lead to drug-susceptible TB being treated with one or more second-line drugs, i.e., treatment that is generally more toxic, less effective, prolonged and more expensive. Furthermore, salvage treatment tends to be less effective than standard treatment, and there is often a greater risk of default.

Sensitivity

False-negative results of molecular resistance assays can be due to platform characteristics. For example, the previous generation GenoType MTBDR*plus* assay had limited sensitivity for detecting *M. tuberculosis* when used directly on smear-negative, culture-positive sputum.[113] More importantly, test sensitivity depends on the proportion of relevant mutations that are targeted by the assay, which generally declines with the increasing number of different genes and intergenic regions involved in resistance to the drug of interest, either known or unknown.[114] Test sensitivity may also show geographic variations if the proportion of relevant mutations covered by the assay varies between regions.[115] This can be due to associations with the genetic background of the strain,[6,93,116,117] and possibly to differential consequences of muta-

tions for bacterial fitness over time, leading to the predominance of the mutation with the least fitness cost.[118]

Finally, studies reporting frequencies of mutations among phenotypically resistant strains may differ in their selection of isolates and DST used. Together, these factors may cause the sensitivity of specific mutations for phenotypic DST to vary widely (Appendix Table A.4).

Specificity

False-positive results of molecular resistance assays can also be due to platform characteristics.[182] In addition, they may occur due to silent mutations picked up by wt probes included in the assay to cover resistance-conferring mutations that are scattered across a larger genomic region, such as with the *pnc*A gene for resistance to PZA.[61] False-positive results in molecular resistance testing may in fact be truly positive if the reference standard (phenotypic DST) has incomplete sensitivity when identifying resistance.[55]

This indicates a more general problem in the interpretation of genotypic DST results. Resistance mutations have almost always been identified based on comparison with phenotypic DST rather than with clinical outcomes. However, phenotypic DST based on critical concentration testing may correlate poorly with clinical resistance. As mentioned before, DST for EMB, SM and ETH at recommended critical concentrations show poor discrimination between clinically resistant and clinically susceptible isolates.[50] Direct evidence of clinical outcomes is available for only a few resistance mutations (Table).

Prediction of a positive or negative test result

Prediction of test results is generally expressed as positive (PPV) and negative (NPV) predictive values. However, as these predictive values depend not only on sensitivity and specificity, but also on the proportion of patients who have true resistance, PPV and NPV can only be meaningfully interpreted for a given pre-test probability of resistance. For mutations that have 100% specificity, the PPV equals 1 (i.e., a positive test result always means growth at the critical concentration), while the NPV equals 1–sensitivity (e.g., for a test sensitivity of 80%, the NPV for growth at the critical concentration will be 20%). In LPA, the PPV is high for RMP, INH, AMK, KM and SM, as all *rpo*B mutations, the *kat*GS315T mutation, all *inh*A promoter mutations, the *rrs* A1408G mutation, and the *rrs* and *rpsl* mutations covered by LPAs have a specificity of practically 100%. For other drugs, the specificity is less than 100%, and the PPV and NPV will differ. An alternative approach is to express this prediction as positive (LR+) and negative likelihood ratios (LR–). Appendix Table A.4 shows these likelihood ratios for EMB, FQs and injectables based on a meta-analysis of studies that evaluated the Genotype MTBDR*sl* assay.[108] A Cochrane review analysing the diagnostic accuracy of the GenoType MTBDR*sl* assay in detecting second-line anti-tuberculosis drug resistance has recently been published.[8] The pooled sensitivity of the test for the detection of FQ resistance was 83.1% and the pooled specificity was 97.7%; the pooled sensitivity and pooled specificity of the test for injectables were respectively 76.9% and 99.5%.

In conclusion

1 Reported sensitivity and specificity estimates for certain mutations are difficult to interpret for drugs for which there is poor or unproven correlation between phenotypic and clinical resistance (e.g., EMB, Group 4 and 5 drugs).

CONSENSUS RECOMMENDATIONS

1. Should molecular testing for M. tuberculosis *rifampicin resistance using currently available methods be the reference for the diagnostic evaluation of patients with presumptive MDR-/XDR-TB?*

Currently available LPA methods detect mutations in *rpo*B codons 516, 526 and 531. There is a high level of agreement between molecular and phenotypic DST. This is due to the fact that mutations associated with RMP resistance are mainly located in the 81 base-pair (bp) core region of *rpo*B, and mutations outside this region are uncommon. However, clinicians need to be aware that strains carrying rare mutations or mutations outside the conventional hot spots targeted by commercial assays may spread and become prevalent in some settings.

Although they do not cover all mutations involved in RMP resistance, molecular methods for RMP could be considered a standard for the diagnostic evaluation of patients with presumptive MDR-TB. In low MDR-TB prevalence countries, physicians should be aware of possible false-positive resistance results of molecular tests, and RMP resistance should be confirmed by a second molecular test on a different sample or by phenotypic tests.

Agreed: 12; disagreed: 0; abstained: 1.

2. Is there value in molecular testing for M. tuberculosis *isoniazid resistance using currently available methods for the diagnostic evaluation and selection of drug regimen of patients with presumptive MDR-/XDR-TB?*

Although >90% of RMP-resistant strains are also resistant to INH, molecular testing for INH drug resistance is important. First, it offers the possibility to add INH to a second-line drug regimen in the absence of a *katG315* mutation. Second, the implications of RMP resistance are different if accompanied by INH resistance.

Table Clinical implications of mutations detected by molecular methods
A)

Mutation	Drug*		Association with in vitro phenotypic resistance	Association with clinical resistance	Frequency among strains categorised as resistant on the basis of critical concentration testing‡
	INH	ETH			
*kat*G S315T	−	+	S315T confers high-level INH resistance (MIC >1 mg/l), but does not affect susceptibility to ETH[69,70,119–121] Note: there are additional mutations in *inh*A or *eth*A, which confer ETH resistance[122]	Indirect evidence strongly suggests that high-level resistance affects clinical outcomes. *kat*G S315T mutations are associated with multidrug resistance (see e.g.[119]). Limited data on direct association between *kat*G S315T mutation and clinical outcome suggest increased risk of first-line treatment failure, death and relapse[123,124]	In a systematic review of 52 studies, 5–98% of INH-resistant isolates showed *kat*G S315T mutations (median 64%, interquartile range 54–79) (Hooijer et al. unpublished)
*inh*A −16G −15T −8A/C	+	−	*inh*A promoter mutations confer low-level INH resistance (MIC <1 mg/l), but significantly affect ETH susceptibility[69,70,119–121,123] Note: there are additional mutations in the structural *inh*A gene, which together with *inh*A promoter mutations result in INH MIC levels >1 mg/l[125]	Limited direct and indirect data, suggesting no effect on cure rates for standard first-line treatment.[123,124] One study showed increased relapse rates with INH-EMB (6 months) in the continuation phase;[138] *inh*A promoter mutations are not associated with multidrug resistance when compared to the *katG* S315T mutation,[119] but have been associated with XDR-TB in South Africa[126]	In various studies, 12–42% of INH-resistant isolates had *inh*A promoter region mutations[113,119,127–130]
	RMP	RBT			
*rpo*B S531L H526mut	−	−	S531L and H526D/Y confer high-level resistance to all rifamycins.[131–136] In contrast, mutation H526L (and possibly H526N/S) only confer low-level resistance to RMP	Strong direct and indirect evidence for association with clinical resistance[128]	More than 95% of RMP-resistant isolates have mutations in the 81-bp core region of the *rpo*B gene. Most studies showed mutations in codons 531 and 526 in 40–65% and 10–40% of RMP-resistant isolates, respectively[127,129,137]
D516mut	−	+	D516mut predominantly affects RMP, but much less so RBT; RBT is still an option for combination chemotherapy[132–134,136,138]		Most studies showed codon 516 and 533 mutations in 5–32% and 2–5% of RMP-resistant isolates, respectively.[127,129,137] Their frequencies are probably underestimated, as low-level resistant isolates may be tested as phenotypically susceptible[67]
L533mut	+	+	L533mut affects susceptibility to all rifamycin only slightly; RMP and RBT are still an option for combination chemotherapy[134,139–141]		
I572F	−	−	I572F mutations are outside the 81-bp core region[142,143–145]	Some studies suggested a role for this mutation in RMP resistance[146,147]	Among the isolates obtained from patients who did not respond to the anti-tuberculosis treatment, some isolates showed mutation at codon 572.[148] Cross-resistance to RBT has been described in one study[149]
	EMB				
*emb*B M306mut	+		M306mut mostly confers low- to moderate-levels of drug resistance, the clinical implications of which are not clear[69,70,150–154]	There have been no studies of the direct effect of *emb*B306 mutations on clinical resistance	In various studies, 20–88% of EMB-resistant isolates had *emb*B306 mutation[108,127,155–159]

Table (continued)
B)

	Drug*			Association with in vitro phenotypic resistance	Association with clinical resistance	Frequency among strains categorised as resistant on the basis of critical concentration testing‡
	KM	AMK	CPM			
rrs A1408G† (1401)	–	–	+	A1408G confers high-level resistance to both KM and AMK, but only low-level CPM resistance; CPM is still an option for combination chemotherapy[46,152,160-163] Note: cave additional mutations in *tlyA* which in conjunction with A1408G confer high-level CPM resistance (unpublished data)	The *rrs* A1401G mutation was associated with clinical resistance to KM[164]	In a systematic review of 22 studies, the A1401G mutation was present in 78% of AMK-resistant and in 76% of CPM-resistant isolates, but in only 56% of KM-resistant isolates[94]
C1409T† (1402)	–	+	–	C1409T confers high-level CPM resistance and low- to intermediate-level KM resistance, but has little effect on AMK susceptibility; AMK is still an option for combination chemotherapy[46,165,166]	There have been no studies of the direct effect of *rrs* 1402 or *rrs*1484 mutations on clinical resistance	In a systematic review of 22 studies, *rrs* C1402T and G1484T mutations were rare (0–2% each) among isolates resistant to any of the injectables[94]
G1491C/T† (1484)	–	–	–	G1491C/T confers high-level AMK, KM and CPM resistance[46,165,166]		
eis G-37T C-12T G-10A	–	+	+	*eis* mutations confer low-level KM resistance.[167] C-14T may confer low-level resistance to both KM and AMK[129]	There have been no studies of the direct effect of *eis* mutations on clinical resistance	In a systematic review of 22 studies, 22% of the KM-resistant isolates harboured the G-10A mutation, 11% the C-14T and 5% the G-37T mutation.[35] In another study, the C-12T mutation was present in 13% of the KM-resistant isolates.[129] C-14T mutation was also associated with AMK resistance[129]
C-14T	–	–	+			
		SM				
*rps*L K43R		–		*rpsL* K43R, *rpsL* K88Q/R and *rrs* A523C and C526T confer moderate- to high-level SM resistance[95,168,169]	There have been no studies of the direct effect of *rps*L *or rrs* mutations on clinical resistance to SM	In various studies, 24–89% of SM-resistant isolates had *rps*L43 mutations.[95,116,159,170] *rps*L88 mutations have been found in 5–27% of SM-resistant isolates, but this prevalence may be lower depending on the geographical setting.[171–173] Together and on average, *rps*L and *rrs* mutations are found in from 75% to over 90% of SM-resistant isolates[128]
K88Q/R		–				
rrs A523C† (514) C526T† (517)		–				
		MFX	OFX			
*gyr*A D94mut		–	–	Mutations in *gyr*A affect MFX and OFX susceptibility. Mutations of residue D94 confer clinical resistance; mutations affecting codon A90 are discussed controversially[174–179] For mutations of codon S91, few data are available, most likely similar to mutations of residue A90[45]	Strong indirect and some direct evidence for association of *gyr*A codon 94 mutations with clinical resistance to OFX[128,164]	D94mut and A90mut in 40–58% and 20–30%, respectively, of OFX- or MFX-resistant isolates[127,129,180,181]
A90mut		+	+			

*– = high-level resistance; the drug should not be given; + = drug susceptibility is not affected or low-level resistance, the drug is an option for combination chemotherapy, in particular when other options are limited due to scarce availability of active compounds.
† *Escherichia coli rrs* nomenclature; the homologous *M. tuberculosis* position is given in brackets.
‡ Note that critical concentration testing uses the ECOFF value to categorise clinical isolates as susceptible. Growth at the critical concentration does not necessarily imply clinical resistance, as it does not define the quantitative level of resistance, i.e., it does not differentiate between low- and high-level 'resistance'.
INH = isoniazid; ETH = ethionamide; MIC = minimum inhibitory concentration; EMB = ethambutol; XDR-TB = extensively drug-resistant tuberculosis; RMP = rifampicin; RBT = rifabutin; bp = base pair; KM = kanamycin; AMK = amikacin; CPM = capreomycin; MFX = moxifloxacin; OFX = ofloxacin; ECOFF = epidemiological cut-off.

Agreed: 13; disagreed: 0; abstained: 0.

3. When should the evaluation for the presence of second-line drug resistance by molecular methods be considered in patients with a presumptive or confirmed diagnosis of tuberculosis?

In all patients with evidence of *M. tuberculosis* with an *rpo*B mutation in a direct specimen or when DST indicates MDR-TB, molecular testing for second-line resistance should be undertaken to guide treatment and to reduce the time to diagnose XDR-TB.

Agreed: 13; disagreed: 0; abstained: 0.

4. What molecular resistance testing results on rifampicin should influence treatment decisions?

More than 95% of RMP-resistant isolates have mutations in the 81-bp core region of the *rpo*B gene. S531L and H526Y/D confer high-level resistance to all rifamycins, with strong direct and indirect evidence of association with clinical resistance. In contrast, D516mut predominantly affects RMP, but much less rifabutin (RBT). RBT could still therefore be considered as an option for combination chemotherapy, although clinical data for the use of RBT in this setting are lacking. As L533mut has only a slight effect on susceptibility to all rifamycins, RMP and RBT are an option for combination chemotherapy for corresponding isolates.

Agreed: 13; disagreed: 0; abstained: 0.

5. What molecular resistance testing results on isoniazid should influence treatment decisions?

The currently available LPA methods detect mutations in *inh*A positions –16, –15 and –8, and *kat*G codon 315. Mutation S315T confers high-level INH resistance (MIC > 1 mg/l), but does not affect susceptibility to ETH. Indirect evidence strongly suggests that high-level resistance affects clinical outcomes. The limited data on the direct association between *kat*G S315T mutation and clinical outcome suggest increased risk of first-line treatment failure, death and relapse. In the case of *katG* S315T mutation, INH should therefore be excluded from treatment.

Compared to *kat*G S315T, *inh*A promoter mutations confer low-level INH resistance (MIC < 1 mg/l), but significantly affect ETH susceptibility. Limited direct and indirect data suggest no effect on cure rates for standard first-line treatment. In the case of *inh*A promoter mutations, INH—preferably in high doses (15–20 mg/kg body weight)—may be administered in combination with other drugs. In the case of *inh*A promoter mutation, the level of resistance should be confirmed by phenotypic methods.

Agreed: 13; disagreed: 0; abstained: 0.

6. What molecular resistance testing results on pyrazinamide should influence treatment decisions?

Unfortunately, until recently, no commercial molecular methods have been able to detect PZA mutations. Mutations associated with resistance can be detected by sequencing the *pnc*A gene.

Agreed: 13; disagreed: 0; abstained: 0.

7. What molecular resistance testing results on ethambutol should influence treatment decisions?

Mutations in *emb*CAB have been detected in resistant strains, with *emb*B306 the codon most commonly affected. M306mut mostly confers low to moderate levels of drug resistance; 20–88% of EMB-resistant isolates had *emb*B306 mutations. These low sensitivity values may be due to the presence of mutations in codons other than *emb*B306, which are not explored by LPAs. These mutations have been located in *emb*B codons 319, 406 and 497, and also in the *emb*C and *emb*A genes. Physicians must be aware of possible false-negative results of molecular tests; in addition, EMB resistance should be confirmed by phenotypic methods. Furthermore, as *emb*B306 mutations have been detected in MDR-TB isolates that are susceptible to EMB, 'false' EMB resistance results may be obtained by molecular tests.

The clinical implications of EMB resistance, which is mostly low or moderate, are not clear at present, nor are those of *emb*B mutations.

Agreed: 13; disagreed: 0; abstained: 0.

8. What molecular resistance testing results on aminoglycosides/polypeptides should influence treatment decisions?

Partial cross-resistance between KM, AMK and CPM has been reported. The *rrs* A1401G mutation is most frequent, and confers high-level resistance to both KM and AMK, but only low-level CPM resistance; CPM may still be an option for combination chemotherapy. C1402T confers high-level CPM resistance and low- to intermediate-level KM resistance, but there is little effect on AMK susceptibility; AMK is therefore still an option for combination chemotherapy. G1484C/T confers high-level AMK, KM and CPM resistance. *rrs* C1402T and G1484T mutations are rare (0–2% each) among isolates resistant to any of the injectables.

Mutations in the *eis* promoter region confer low levels of resistance to KM, and possibly AMK. In these cases, LPA tests that do not explore this region present reduced sensitivity in detecting resistance to these drugs. The clinical significance of these low-level resistance mutations is unclear.

Agreed: 13; disagreed: 0; abstained: 0.

9. What molecular resistance testing results on fluoroquinolones should influence treatment decisions?

LPAs are relatively specific; however, their unsatisfactory sensitivity affects molecular testing for resistance to FQs. Mutations in *gyr*A affect MFX

and ofloxacin (OFX) susceptibility. D94mut and A90mut have been detected in respectively 40–58% and 20–30% of OFX- or MFX-resistant isolates. Treatment with FQs should be excluded when *gyr*A D94mut is detected. For mutations affecting codon A90, the clinical implications are less clear; for mutations in codon S91, few data are available, but these are most likely similar to mutations of residue A90. Current LPA methods detect mutations in codons 80–81 and 88–95 of *gyr*A. Discordance between LPA and phenotypic DST results may therefore be due to mutations in other *gyr*A gene regions or in *gyr*B. Clinicians should be aware of possible false-negative results of molecular tests, and FQ susceptibility should be confirmed by phenotypic methods.

Agreed: 13; disagreed: 0; abstained: 0.

10. What should be the consequences of the evaluation for the presence of second-line drug resistance by molecular methods in patients with a presumptive or confirmed diagnosis of tuberculosis pending the results of drug susceptibility testing in solid or liquid culture media?

While the results of phenotypic second-line drug resistance testing are pending, physicians should be guided by the principles of investigating molecular DST results for RMP, INH, EMB, FQs and injectable agents (as outlined in this statement) in their initial choice of a second-line anti-tuberculosis drug regimen.

Agreed: 12; disagreed: 1; abstained: 0.

11. Can treatment recommendations be provided based on the molecular drug susceptibility testing results of any other available drugs (delamanid, bedaquiline, prothionamide/ethionamide, cycloserine/terizidone, PAS, meropenem/imipenem, clofazimine, linezolid)?

Current molecular methods do not detect mutations related to resistance of these drugs. However, *inhA* promoter mutations significantly affect ETH/PTH (see answer to 'What molecular resistance testing results on isoniazid should influence treatment decisions?')

Agreed: 13; disagreed: 0; abstained: 0.

12. Should molecular testing for M. tuberculosis *drug resistance be performed by targeted diagnoses (LPAs, Xpert) or by whole genome sequencing)?*

While LPAs and other technologies (e.g., Xpert) are apparently limited in their ability to provide comprehensive information on genomic mutations that confer bacterial drug resistance, WGS provides the complete sequence information of the bacterial genome. However, due to the lack of correlation with in vitro (phenotypic DST) and in vivo (treatment outcome) data at present, it is not possible to interpret the clinical value of the vast majority of mutations or polymorphisms detected. Systematic data collection and correlation of WGS data with in vitro DST and clinical outcomes will be required to assess the added clinical value of this method over existing technologies.

Agreed: 13; disagreed: 0; abstained: 0.

13. If the results of molecular and culture-based drug susceptibility testing differ, what is the gold standard?

The level of discordance between molecular and culture-based DST depends on the drug and the genomic region evaluated. Despite the fact that results of phenotypic methods do not always correspond to response to clinical treatment, culture-based methods are still regarded by most experts involved in this document as the gold standard for DST.

Agreed: 13; disagreed: 0; abstained: 0.

14. How should the results of molecular drug susceptibility testing be reported by the laboratory to the clinicians?

Whenever molecular testing allows, results should always be reported with the specific mutation detected and a description of the clinical implications of the presence of the mutation, as outlined in the Table.

Agreed: 13; disagreed: 0; abstained: 0.

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Due to the slow growth rate of *M. tuberculosis*, culture-based DST results are not readily available to treating physicians to guide the initial decision regarding the choice of treatment for MDR-TB patients. Molecular methods are revolutionising the management of drug-resistant TB patients. For several years, the WHO has strongly recommended the use of molecular DST using Xpert for the detection of RMP resistance in individuals presumed to have MDR-TB or HIV-associated TB,[73,101] and recommended the use of commercial LPAs for the rapid diagnosis of MDR-TB.[72] Xpert and LPA results form the basis of clinical decision-making where these technologies have become available.[74,183] This is especially important for the identification of mutations occurring in the *rpo*B gene, resulting in RMP resistance, as RMP is currently the most effective drug for the treatment of TB.[184,185]

Rapid second-line *M. tuberculosis* DST is necessary to tailor anti-tuberculosis treatment regimens for individual patients early after the diagnosis of TB. It was recently identified that 60% of MDR-TB strains of *M. tuberculosis* in the European Region are also resistant to EMB and PZA by phenotypic testing, >30% are resistant to ETH/PTH, >25% are resistant to any WHO Group 2 second-line injectable

drug, and >17% are resistant to any WHO Group 3 FQ.[186] Almost all the XDR-TB strains of *M. tuberculosis* in Europe are also resistant to PZA and EMB.[187]

At present, the WHO does not advocate the use of molecular DST for second-line anti-tuberculosis drugs by either LPAs or WGS. However, there is growing evidence that molecular DST can be a reliable method for the rapid identification of genomic mutations in *M. tuberculosis*, e.g., to detect mutations that lead to drug resistance to WHO Group 2 drugs (second-line injectable drugs) or WHO Group 3 drugs (FQs), providing the potential for individualising anti-tuberculosis treatment at the start of treatment. There is still a caveat not to ignore phenotypic DST, as LPAs still frequently miss drug resistance mutations. False-positive results are very uncommon in molecular DST.[8] Novel technologies such as NGS allow rapid identification of clinical relevant mutations not yet detected by Xpert or LPAs.[142,188]

For the first time, this TBNET/RESIST-TB statement provides a consensus of clinicians, molecular biologists and microbiologists on the interpretation and reporting of the specific genetic results of molecular DST to guide the management of patients with drug-resistant TB. Basing treatment decisions on the results of molecular DST has been common practice for physicians caring for HIV-infected patients for more than a decade,[189,190] and this is now becoming important for physicians caring for patients with TB as well.

In this rapidly evolving field, the present consensus recommendations from this document are only a snapshot in time, and such recommendations will need to be updated on a regular basis. In the future, reporting molecular DST results by laboratories should go beyond 'susceptible' and 'resistant' and list identified mutations to provide guidance for physicians according to the best available evidence. As molecular technologies are further developed, it will be important to match information about molecular DST results, quantitative measures of phenotypic drug resistance and clinical outcome in quality controlled databases.[191] This will be important not only for mutations with known clinical relevance, but also for the great majority of mutations identified by WGS with unknown significance.[192–195]

Synergistic analysis of mutations in the *M. tuberculosis* genome, phenotypic DST results and information on clinical outcome will substantially improve the treatment of patients with drug-resistant TB. If quality-assured data can be collected systematically and the results are reliable and reproducible, the growing evidence on the significance of specific mutations in the *M. tuberculosis* genome may ultimately allow molecular diagnostics to replace culture-based anti-tuberculosis DST.

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APPENDIX

Table A.1 Anti-tuberculosis drugs, their mechanism of action, resistance mechanism and function

Drug	Mechanism of drug action	Resistance mechanism	Function of gene product	Comments
Group 1 drugs				
EMB	Interferes with cell wall synthesis	*emb*A, *emb*B	Arabinosyl transferase	
INH	Interferes with mycolic acid synthesis	*kat*G, *inh*A	Catalase/peroxidase (katG), enoyl reductase (inhA)	*inh*A mutations confer low grade phenotypic resistance
PZA	Unclear	*pcn*A	Pyrazinamidase	
RMP/RBT/RPT	Inhibits RNA polymerase	*rpo*B	RNA polymerase (beta subunit)	Cross-resistance between members of this family
Group 2 drugs				
AMK	Inhibits protein synthesis	*rrs, eis*	16S rRNA (*rrs*), aminoglycosidase acetyltransferase (*eis*)	KM, AMK and CPM partly show cross-resistance depending on the gene and mutation involved
CPM/viomycin	Inhibits protein synthesis	*rrs, tly*A	16S rRNA (*rrs*), rRNA methyltransferase (*tly*A)	
KM	Inhibits protein synthesis	*rrs, eis*	16 rRNA (*rrs*), aminoglycosidase acetyltransferase (*eis*)	*eis* mutations confer low grade resistance towards KM
SM	Inhibits protein synthesis	*rps*L, *rrs, gid*B	S12 ribosomal protein (*rps*L), 16S rRNA (*rrs*), guanosine methyltransferase (*gid*B)	*gid*B mutations confer low grade resistance towards SM
Group 3 drugs				
LFX/OFX/MFX	Interferes with mycobacterial topoisomerase	*gyr*A, *gyr*B	DNA gyrase	Other mechanisms are thought to exist but have not been identified
Group 4 drugs				
CS/terizidone	Inhibits peptidoglycan synthesis (presumably interferes with synthesis of D-ala-D-ala)	Unknown	Unknown	Unknown
ETH/PTH	Inhibits mycolic acid synthesis	*inh*A *eth*A	Enoyl reductase	Cross-resistance with INH (*inh*A)
PAS	Interferes with folate metabolism	*thy*A *ribD* *fol*C	Thymidylate synthase A Dihydrofolat reductase Dihydrofolat synthase	Other mechanisms of resistance may exist
Group 5 drugs				
Amoxicillin plus clavulanate	Interfers with peptidoglycan synthesis	Unknown	Unknown	
CLM	Inhibits protein synthesis	*erm*	23S rRNA methylase	*M. tuberculosis* has inducible *erm* methylase
CFZ	Unknown	Rv 0678	Transcriptional repressor of MmpS5-MmpL5 efflux pump	Mutations confer cross-resistance to bedaquiline
Linezolid	Inhibits protein synthesis	*rpl*C, *rrl*	Ribosomal L3 protein, 23S rRNA	
Meropenem plus clavulanate	Interferes with peptidoglycan synthesis	Unknown	Unknown	
Thioacetazone	Unknown	Unknown	Unknown	
New drugs				
Apramycin	Inhibits protein synthesis	*rrs*	16S rRNA (*rrs*)	
Bedaquiline	Inhibition of ATP synthase	*atp*E, Rv0678	ATP synthase, transcriptional repressor (Rv0678) of MmpS5-MmpL5 efflux pump	Mutations of Rv0678 mediate cross-resistance to CFZ
Delamanid/PA-824	Inhibits cell wall synthesis	*ddn*	Deazaflavin-dependent nitroreductase	
		fdG1	Glucose-6-phosphate dehydrogenase	
		*fbi*A, *fbi*B, *fbi*C	Synthesis of deazaflavin cofactor F420	
Spectinamides	Inhibits protein synthesis	*rrs*	16S rRNA (*rrs*)	
Sutezolid/AZD-5847	Inhibits protein synthesis	*rrl*	23S rRNA	

EMB = ethambutol; INH = isoniazid; PZA = pyrazinamide; RMP = rifampicin; RBT = rifabutin; RPT = rifapentine; AMK = amikacin; KM = kanamycin; CPM = capreomycin; SM = streptomycin; LFX = levofloxacin; OFX = ofloxacin; MFX = moxifloxacin; ETH = ethionamide; PTH = prothionamide; PAS = para-aminosalicylic acid; CLM = clarithromycin; CFZ = clofazimine; ATP = adenosine triphosphate.

Table A.2 Updated WHO critical concentrations for elected first- and second-line agents for the treatment of tuberculosis[57,58]

Drug	Löwenstein-Jensen μg/ml	Middlebrook 7H10 μg/ml	Middlebrook 7H11 μg/ml	MGIT 960 μg/ml
Rifampicin	40.0	1.0	1.0	1.0
Isoniazid	0.2	0.2	0.2	0.1
Pyrazinamide	—	—	—	100.0
Ethambutol	2.0	5.0	7.5	5.0
Streptomycin	4.0	2.0	2.0	1.0
Kanamycin	30	5.0	6.0	2.5*
Amikacin	30*	4.0*	—	1.0
Capreomycin	40	4.0*	—	2.5
Ofloxacin	4.0*	2.0	2.0	2.0
Moxifloxacin	—	0.5*	—	0.5*†
		2.0*		2.0*

* Suggested updates from reference 58; not yet formally published by the WHO.
† Proxy for ofloxacin in case ofloxacin is not tested.
WHO = World Health Organization; MGIT = Mycobacteria Growth Indicator Tube.

Table A.3 Hot spot of *rpo*B gene: result of commercial LPA tests in the presence of mutations in specific codons, codons known to host silent mutations, mutations associated with susceptible RMP result in the phenotypic MGIT DST*

Codon	INNO–LiPA®	GenoType®	AID TB Resistance	Silent mutation	MGIT-S†
505	—	W1−	—		
506	—	W1−	—		
507	—	W1−	—		
508	W1−	W1−	—	T508	
509	W1−	W1−	—		
510	W1−	W2−	—	Q510	
511	W1−	W2−	—	L511	Gln/Pro
512	W2−	W2−	—		Arg
513	W2−	W2− W3−	W1−	Q513	
514	W2−	W3−	W1−	F514	
515	W2−	W3−	W1−		
516 Val	W2− M2+	W3− W4− M1+	W1− M1+		Val
516 Tyr	—	—	W1− M1+		
516 other	W2−	W3− W4−	W1− M1+		Phe
517	W2−	W4−	W1−		
518	W3−	W4− W5−	—		
519	W3−	W4− W4−	—		
520	W3−	W4−	—		
521	W3−	W4−	—		
522	W3−	W4− W6−	—		Gln
523	W4−	W6−	W2−		
524	W4−	W6−	W2−	T524	
525	W4−	W6−	W2−		
526 Tyr	W4− M4a+	W7− M2a+	W2−		
526 Asp	W4− M4b+	W7− M2b+	W2− M2+		
526 Arg	—	—	W2− M2+		
526 other	W4−	W7−	W2− M2+		Asn/Cys/ Leu/Ser
527	W4−	W7−	W2− M2+		
528	W5−	W7−	W2−		
529	W5−	W7−	—		
530	W5−	W8−	—		
531 Leu	W5− M5+	W8− M3	W3−		
531 Trp	—	—	W3− M3+		
531 other	W5−	W8−	W3−		Tyr
532	W5−	W8−	W3− M3+	A532	
533	W5−	W8−	W3− M3+	L533	Arg/Pro‡
534	—	W8−	W3−		

* W = wild type probe; M = mutated probe; S = codon in which silent mutations have been reported. Some of the codons that could be clinically relevant, such as V146F and I572F, are not included in LPAs.
† Mutations reported associated to susceptible RMP result in the phenotypic MGIT DST.
‡ 533P can be missed by LPA.
LPA = line-probe assay; MGIT = Mycobacteria Growth Indicator Tube; DST = drug susceptibility testing; RMP = rifampicin.

Table A.4 LR+ and LR– for resistance to EMB, FQs and injectables (GenoType® MTBDR*s*l assay)*

Drug	Sensitivity % (95%CI)	Specificity % (95%CI)	LR+ % (95%CI)	LR– % (95%CI)
EMB	67.9 (65.2–70.6)	79.9 (77.3–82.3)	4.879 (2.250–10.581)	0.498 (0.383–0.648)
FQs	87.4 (84.5–89.9)	97.1 (96.1–98.0)	26.368 (12.851–54.102)	0.182 (0.109–0.303)
Amikacin	82.6 (77.7–86.9)	99.5 (98.7–99.8)	68.851 (7.845–604.234)	0.192 (0.150–0.245)
Kanamycin	44.4 (39.6–49.2)	99.3 (98.5–99.7)	48.693 (7.289–325.260)	0.561 (0.430–0.732)
Capreomycin	82.0 (77.2–86.2)	97.3 (96.3–98.1)	18.211 (9.964–33.285)	0.151 (0.037–0.609)

* Reproduced from reference 108.
LR = likelihood ratio; + = positive; – = negative; EMB = ethambutol; FQ = fluoroquinolone; CI = confidence interval.

RESUME

L'émergence de souches de *Mycobacterium tuberculosis* pharmacorésistantes défie la lutte contre la tuberculose (TB) dans le monde. Bien que les méthodes basées sur la culture aient été considérées comme l'étalon or des tests de pharmacosensibilité (DST), les méthodes moléculaires fournissent des informations rapides sur les mutations du génome de *M. tuberculosis* associé à la résistance aux médicaments antituberculeux. Nous avons obtenu un consensus sur l'utilisation des résultats des DST moléculaires pour les décisions relatives au traitement clinique des patients tuberculeux. Ce document a été élaboré par TBNET et RESIST-TB afin d'atteindre un consensus sur les standards de rapports de l'utilisation clinique des résultats des DST moléculaires. La revue de la littérature disponible et le recherche de preuves a inclus la recherche manuelle de revues médicales et la recherche dans les bases de données électroniques. Le panel a identifié des mutations isolées d'un seul nucléotide dans les régions génomiques de *M. tuberculosis* codant pour *kat*G, *inh*A, *rpo*B, *emb*B, *rrs*, *rps*L et *gyr*A, qui sont probablement liées à la pharmacorésistance in vivo. L'identification de l'une quelconque de ces mutations dans des isolats cliniques de *M. tuberculosis* a des implications en termes de prise en charge des patients tuberculeux, dans l'attente des résultats des DST in vitro. Cependant, l'interprétation est compliquée par des résultats faussement positifs et négatifs dans la détection des mutations associées à la résistance aux médicaments. En effet, il y a une corrélation médiocre ou non démontrée entre la pharmacorésistance phénotypique et clinique. En conséquence, les rapports relatifs aux résultats des DST moléculaires devraient inclure des informations spécifiques sur les mutations identifiées et fournir une guidance aux cliniciens dans l'interprétation et le choix du protocole thérapeutique initial approprié.

RESUMEN

La aparición de cepas de *Mycobacterium tuberculosis* resistentes a los fármacos anti-tuberculosos representa un reto para el control global de la tuberculosis (TB). Aunque los métodos basados en los cultivos han sido considerados como el método de referencia para el estudio de la susceptibilidad a los fármacos (DST), los métodos moleculares proveen de una información rápida de la presencia de las mutaciones asociadas a resistencia a estos fármacos. TBNET y RESIST-TB han elaborado este documento de consenso para la interpretación de los resultados moleculares de detección de resistencias en la toma de decisiones terapéuticas en los pacientes con TB. La revisión de la bibliografía disponible y la búsqueda de evidencia se ha realizado mediante búsqueda manual en las publicaciones científicas y búsqueda electrónica en las bases de datos. El grupo de trabajo ha identificado mutaciones puntuales en regiones genómicas de *M. tuberculosis* en *kat*G, *inh*A, *rpo*B, *emb*B, *rrs*, *rps*L y *gyr*A, que están relacionadas con resistencia in vivo a los fármacos antituberculosos. Mientras se dispone de los resultados fenotípicos, la detección de estas mutaciones en los aislados clínicos de *M. tuberculosis* tiene implicaciones en el manejo de los pacientes con TB. Sin embargo, la existencia de resultados falsos positivos y negativos al detectar mutaciones con muy poca o sin una demostrada correlación entre resistencia clínica y fenotípica, complica la interpretación. Como consecuencia de ello, los resultados de las técnicas de detección molecular de resistencias deben incluir información específica de las mutaciones identificadas y proveer pautas para los clínicos en la interpretación y en la elección del régimen antibiótico inicial apropiado.