Issues in *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing: Rifampin

HIGHLIGHTS

- Rapid and reliable detection of rifampin (RIF) resistance is critical for the diagnosis and treatment of drug resistant and multidrug-resistant tuberculosis (MDR TB).
- Most RIF resistant clinical *Mycobacterium tuberculosis* complex (MTBC) isolates contain mutations in the *rpoB* gene with 96% occurring within the Rifampin Resistance Determining Region (RRDR).
- For the majority of *rpoB* gene mutations, a high correlation with growth-based drug susceptibility testing (DST) is found. Results from molecular assays can be discordant with growth-based testing results in some cases, therefore DNA sequencing should be performed to confirm mutations identified when a non-sequence based molecular assay is utilized.
- A subset of isolates with mutations in the RRDR that are susceptible to RIF using growth-based DST methods (or have only slightly elevated MIC values) are considered to have low-level resistance to RIF.

BACKGROUND

Rifampin (RIF), the most commonly used member of the rifamycin drug class, is the cornerstone of the first-line regimen used for treatment of drug susceptible *Mycobacterium tuberculosis* complex (MTBC) due to its potent bactericidal activity. RIF can kill MTBC rapidly by inhibiting the DNA dependent RNA polymerase β-subunit encoded by the *rpoB* gene. The effectiveness of treatment for tuberculosis depends on many factors including the bacterial strain, its metabolic activity, the bacterial burden, pharmacodynamics of the drugs, the site of infection, and the compliance of the patient. Different drugs in a treatment regimen are believed to act on different populations of bacteria in the lesions. RIF is most effective against the dormant bacteria that have short spurts of metabolic activity or active growth because it can kill within 15-20 minutes unlike other commonly used drugs.[1]

RIF kills MTBC by inhibiting the elongation of messenger RNA, ultimately halting transcription. Mutations in the MTBC *rpoB* gene cause changes in the structure of the β-subunit which can lead to various degrees of sensitivity to RIF.[2–4] The majority of RIF resistant clinical MTBC isolates contain mutations in the *rpoB* gene.

Wild-type strains of MTBC which have not been exposed to RIF are highly susceptible, with minimal inhibitory concentrations (MIC) in the range of 0.125 µg/mL to 0.5 µg/mL in the BACTEC™ MGIT™ 960 system (Becton Dickinson). Resistant strains tend to have much higher MICs, most commonly around 8 µg/mL or as high as 50 µg/mL.[5,6] Therefore, in the majority of cases, a test concentration of 1 µg/mL allows discrimination between the majority of susceptible and resistant strains.[7] However, in March 2021, the World Health Organization (WHO) updated the critical

The World Health Organization (WHO) has published a "Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance" (2021). This is the first document to provide a common, standardized reference for the interpretation of resistance to first and second-line drugs. The catalogue includes 17,000 mutations, their frequency and association with resistance. The mutations are further classified into two tiers. Tier 1 comprises genes considered most likely to contain the resistance mutations. Tier 2 comprises genes that are reasonably likely to contain resistance mutations, with the additional, literature-defined promoter sequences. The WHO document is an important resource with detailed information on numerous mutations associated with drug resistance and may include mutations not specifically addressed in this white paper. We recommend readers to review both documents if they are interested in molecular detection of drug resistance.

concentration for rifampin (7H10/MGIT) by lowering it to 0.5 µg/mL (from 1.0 µg/mL).[8]

In addition to traditional growth-based drug susceptibility testing (DST), molecular detection of mutations can provide valuable information to predict drug resistance, particularly for RIF. Mutations that alter the amino acid sequence—also known as non-synonymous mutations—in the 81-bp "hot spot" known as the RIF resistance determining region (RRDR) of the *rpoB* gene are almost always associated with resistance.[9,10] It has been reported that collectively 96% of RIF resistant MTBC strains have a mutation within the RRDR of the *rpoB* gene with a limited number of mutations outside the RRDR conferring resistance to RIF.[9,11]

Historically, mutations in the rpoB gene of MTBC have been referenced by a numbering system based on the *E. coli genome*. There has been an international movement to change the numbering system to be based on the *M. tuberculosis* (H37Rv) reference genome.[12] We will use MTBC numbering throughout this document (**Figure 1**).

However, there are isolates with non-synonymous mutations in the RRDR which are susceptible to RIF by use of growth-based DST methods or have only slightly elevated MIC values close to that of the critical concentration used for testing. Isolates with these results are considered to have low-level resistance to RIF. The clinical impact of these mutations, also referred to as "disputed "or "discordant" mutations, are not fully understood, but there is evidence in the literature that these mutations can be associated with treatment failure.[13,14] Examples of mutations conferring low-level RIF resistance include: Leu430Pro, Asp435Tyr, His445Asn, His445Leu, His445Cys, Leu452Pro and Ile491Phe (formerly codons 511 516, 526, 533, and 572, respectively, using the *E. coli* numbering system).[8,15]

As mentioned previously, there are RIF resistant isolates that have mutations outside of the RRDR. Therefore, molecular approaches that do not assess mutations outside of the RRDR, such as codons 170, 250, 299, 482 and 491,[16] may be insufficient to detect all molecular markers of RIF resistance. An additional challenge with molecular detection of resistance is that a strain may be identified as RIF resistant due to a mutation that does not alter the amino acid—also known as a synonymous mutation or silent mutation—in the RRDR, which does not confer resistance.

Case studies described in the literature report similar rates of treatment failure in patients receiving RIF therapies that were infected with strains containing certain *rpoB* mutations associated with low-level resistance described above as compared to patients infected with strains containing mutations that confer high-level RIF resistance.[17] Of note, there is considerable variability of growth-based DST results with MTBC strains containing these *rpoB* mutations associated with low-level RIF resistance, as they can often test as susceptible, especially in automated liquid culture systems.[13,14,18] Expert consultation should be sought to consider the treatment regimen for these patients.

Figure 1. Alignment of 81-bp RRDR of *E. coli* and *M. tuberculosis* complex

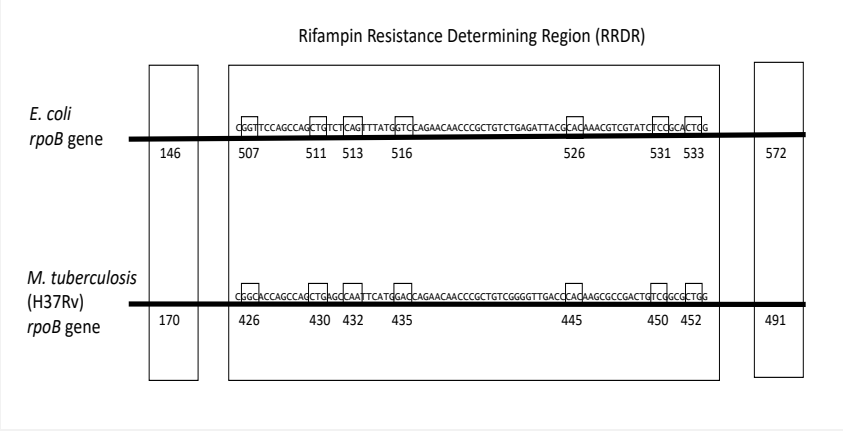
The *rpoB* mutation is now named using the *Mycobacterium tuberculosis* codon system.

Converting from *E. coli* to MTBC within the RRDR: Subtract 81 from the old/*E.coli* codon number to obtain the new/MTBC codon number.

For Example: Codon 526-81= 445 in the new/MTBC numbering scheme.

Converting from *E. coli* to MTBC outside the RRDR: This is not a simple conversion as above. The equivalent codon to *E. coli* 146 in *E.coli* is MTBC codon number 170.

The seven codons in the RRDR frequently associated with RIF resistance, using the annotation system specific to each species is shown. Additionally, shown are the two most common mutations found outside of the RRDR. Figure adapted from Andre et al.[12]



PRACTICAL LABORATORY ISSUES

Growth-based Phenotypic RIF DST and Test Methods

Current growth-based DST methods for RIF in the US include the agar proportion (AP) method using the Clinical and Laboratory Standards Institute (CLSI) recommended critical concentrations[19,20] on either 7H10 or 7H11 medium, commercial automated broth systems: BACTEC™ MGIT™ 960 (MGIT) with the BACTEC™ SIRE Drug kit (MGIT Assay, Becton Dickinson) and VersaTREK™ Automated Microbial Detection System (VersaTREK) with the VersaTREK™ Myco Susceptibility kit (VersaTREK Assay, TREK Diagnostic Systems, ThermoFisher Scientific™); and a microdilution plate method, Sensititre™ *Mycobacterium tuberculosis* MIC Plate (ThermoFisher Scientific™) (**Table 1**). Of these, only the MGIT and VersaTREK systems have US Food and Drug Administration (FDA)-cleared assays to detect RIF resistance. The AP method is considered the reference method and the Sensititre™ assay is for research use only (RUO).

The AP method compares the growth of an isolate on solid agar medium with and without drug at various concentrations. An isolate is determined to be resistant if the number of colonies that grow in the presence of the drug is ≥1% of the number of colonies of the same isolate that grows in the absence of the drug. The agar proportion method also allows the determination of the proportion or percentage of resistance organisms within the sample. This method remains the accepted standard and allows for the detection of both susceptible and resistant organisms from a clinical specimen but is not without its challenges.[19] For example, variability among laboratories may be introduced with the production of drug containing agar plates and the oleic albumin dextrose catalase (OADC) commercial growth supplement used in the preparation of the agar medium that may vary in purity from lot-to-lot which impacts drug activity. Few clinical laboratories use the AP method because of the complexity of preparing the drug plates and the long turnaround time compared to commercial automated broth systems.

The commercially available BACTEC™ MGIT™ 960 SIRE™ kit is based on growth of the MTBC isolate in a drug-containing tube compared with a drug-free tube (growth control). The MGIT™ 960 instrument continuously monitors tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tube compared with the fluorescence of the growth control tube is used by the instrument to determine susceptibility results. The VersaTREK™ Myco Susceptibility kit utilizes a standardized suspension of MTBC to inoculate into drug-containing VersaTREK™ Myco bottles and a drug-free control bottle which are monitored for growth. Growth is detected by a change in gas pressure in the headspace of the bottle due to the consumption of oxygen by the mycobacterial isolate. Commercial automated broth systems are the recommended, and most commonly used, method for first-line DST in clinical laboratories, including RIF,[21] because of the shorter incubation times required for obtaining results as compared to AP.

Per the most recent CLSI M24 3rd Edition,[19] critical concentrations for RIF DST are 1.0 µg/mL for 7H10 and 7H11, the MGIT assay and the VersaTREK assay.[19] However, the WHO performed a systematic review which revealed that the critical concentrations for RIF in 7H10 Agar and the MGIT assay have been set too high, which was corrected by lowering the critical concentrations to 0.5 µg/mL.[8] One comparison between MGIT and VersaTREK assays found 100% correlation between the two assays. However, it was noted that when needles are used to inoculate VersaTREK assay bottles there could be a decrease in the possibility of contamination but increase the risk of occupational transmission.[22] The manufacturer also offers a modified VersaTREK assay bottle with a screw cap which eliminates the need for a needle at this step.

One commercial method for the determination of MIC is the Sensititre™ *Mycobacterium tuberculosis* MIC Plate. This method is a broth microdilution assay, consisting of a 96-well microtiter plate containing twelve antimicrobial agents at appropriate dilutions with RIF being tested at a range of 0.12 µg/mL to 16 µg/mL. There are no CLSI established interpretive break points for the assay and the manufacturer does not provide interpretive criteria for RIF, but the MIC is the lowest concentration with no visible growth. The manufacturer's protocol states that growth from 7H10 agar should be used for preparing the inoculum, followed by incubation, and checking for growth at 10 days and plates can be read from 7-21 days post-inoculation. Although the turnaround time for this method is longer than the MGIT assay, it has the advantage of reporting susceptibility results for all 12 drugs at one time with most plates being able to be read from 10-14 days.[23–25]

Molecular-based Genotypic RIF DST and Test Methods

Molecular-based assays provide rapid DST results for RIF compared to growth-based methods. Molecular testing methods for detection of mutations in the RRDR associated with resistance to RIF include the Xpert©MTB/RIF (Cepheid, FDA market authorization), the MTBDR*plus* line probe assay (Hain LifeScience, RUO) and other laboratory developed tests (LDTs) utilizing real-time PCR, targeted and whole genome sequencing methods, or a combination of these. These assays can be performed rapidly and directly on the specimen or on isolates. The incorporation of these into testing algorithms and for patient management has been described.[26,27]

The Xpert® MTB/RIF assay is a sample-to-answer, single cartridge test utilizing molecular beacons to detect MTBC and RIF resistance which includes a sample processing control (SPC) to ensure adequate processing and monitor for the presence of inhibitors. Line probe assays (LiPA) allow for detection of MTBC DNA from isolates or directly from clinical specimens. The procedure involves a DNA extraction step followed by PCR, labeling and hybridization. The GenoType MTBDR*plus* is available for purchase in the US as a RUO product for the identification of MTBC and RIF resistance. This assay is not FDA-cleared and therefore, requires the performance of a validation study prior to adopting the method. The identification of RIF resistance is possible by the detection of the most significant mutations of the *rpoB* gene.

There are two general types of sequencing-based assays: targeted sequencing such as Sanger sequencing, pyrosequencing (PSQ) and targeted next generation sequencing (tNGS) and non-targeted or whole genome sequencing (WGS). The targeted sequencing methods mentioned vary in their methodology, but they can detect specific "targeted" changes in a gene or loci. PSQ and tNGS can be performed on clinical specimens due to the PCR amplification included in the method and isolates whereas whole genome sequencing is most commonly performed on isolates. Depending on workflows, targeted sequencing methods can have a shorter turnaround time and be more cost-effective.

Similarly, with sequence-based methods used as a stand-alone test or to confirm PCR or probe-based testing, the test or analysis pipeline must differentiate between high-confidence mutations likely to be associated with RIF resistance, mutations that may be associated with resistance and those that are known not to be asscociated with resistance.[28–30] The available testing can be divided into growth-based and molecular-based DST methods (**Table 1**).

Table 1. Growth-based and Molecular-based Drug Susceptibility Methods for RIF[a]

Growth-based Method	Determination of Resistance	Commercially Available Test Systems-US (Regulatory Status)
Agar Proportion 7H10	The number of colony forming units (CFU) growing on medium containing RIF at the critical concentration of 1.0 µg/mL compared with the number of CFU growing on drug free medium[b]	N/A
Agar Proportion 7H11	The number of CFU growing on medium containing RIF at the critical concentration of 1.0 µg/mL compared with the number of CFU growing on drug free medium[b]	N/A
Automated Broth System	Growth in the presence of RIF at the critical concentration of 1.0 µg/mL[b]	BACTEC™ MGIT™ 960 SIRE Drug Kit, 1.0 µg/mL (FDA cleared), VersaTREK™ Myco Susceptibility Kit, 1.0 µg/mL (FDA cleared)
Microdilution Plate Method	The lowest concentration (range 0.12-16 µg/mL) that shows no visible growth	Sensititre™ *Mycobacterium tuberculosis* MIC Plate (RUO)[c]
Molecular-based Method	**Determination of Resistance**	
Real-time PCR	Detection of mutations in the RRDR through probe binding	Xpert® MTB/RIF (FDA-market authorized)
Line Probe Assay (LiPA)	Detection of mutations in the RRDR associated with RIF resistance by lack of hybridization to wild-type sequence probes and/or hybridization to probes containing known mutations visualized on a test strip[d]	GenoType MTBDR*plus* (RUO)[c]
Sanger sequencing	Detection of specific mutations within the genetic loci associated with resistance to RIF (*rpoB*).	LDT only
Pyrosequencing (PSQ)	Detection of targeted mutations within the genetic loci associated with resistance to RIF (*rpoB*)	LDT only
Targeted next generation sequencing (tNGS)	Detection of mutations within the genetic loci associated with resistance to RIF (*rpoB*)	Deeplex® Myc-TB (RUO) Ion AmpliSeq™ TB Panel (RUO)[c]
Whole genome sequencing (WGS)	Detection of mutations in *rpoB* and analyzing the entire genome for other genetic predictors of drug resistance	LDT only

a. Critical concentrations included in the table are from CLSI M24 unless otherwise noted.[19]

b. WHO updated the critical concentration for Rifampin by lowering it to 0.5 µg/mL (from 1.0µg/mL).[8]

c. Assays are commercially available as RUO but can be validated for clinical testing.

d. GenoType MTBDR*plus* VER 2.0 also detects specific mutations in *katG* and *inhA*, please review the instructions for use for complete details.

Considerations for RIF DST

Growth-based DST

Growth-based DST with solid and liquid methods has been well studied for RIF, and a consensus based on a robust assessment of published studies, laboratory experience and expert opinions regarding methodology, critical concentrations, and expected performance is established.[7] With all growth-based methods, particularly the automated systems, it is good practice to ensure inocula do not contain clumps of MTBC and to allow sufficient time for settling of clumps in the broth suspension before inoculation of the drug tubes as some reports have shown improvement in reproducibility of results.[31] One complication to the interpretation of DST for RIF is the potential for discrepant results among the increasing number of different DST methods available, including the rapid molecular methods. Additionally, in some settings, detection of RIF resistance serves as a reliable (although not complete) proxy of MDR TB. The advantages of rapidly identifying RIF resistance, particularly through molecular methods, includes earlier identification of patients on inappropriate first-line regimens, rapid screening of patients at risk of MDR TB, and early interruption of MDR TB transmission.[7]

A comprehensive review of studies that compared the MGIT assay with other growth-based methods for detecting resistance to RIF showed excellent (98%) sensitivity for the MGIT assay.[7] However, another study found that the MGIT assay was less sensitive than the Lowenstein Jensen proportion method in detecting RIF resistance.[18]

Molecular-based DST

Molecular-based assays have some associated challenges and issues. For the majority of *rpoB* gene mutations, a high correlation with growth-based DST is found but results from molecular assays can be discordant with growth-based results in some cases. Despite the excellent sensitivity of the Xpert® MTB/RIF assay for smear-positive sputum specimens, it is somewhat less sensitive when testing smear-negative sputum and in some types of extrapulmonary specimens (Xpert® MTB/RIF is not FDA market authorized for these specimen types), which are known to contain lower levels of bacilli than pulmonary specimens. The assay also has limited capacity to detect RIF resistance associated mutations in mixed samples.[32,33]

Another diagnostic challenge and consideration are the identification of synonymous/silent mutations which do not alter the *rpoB* amino acid sequence and are not associated with RIF resistance. However certain methods, such as Xpert® MTB/RIF and MTBDR*plus* line probe assay may identify these mutations and report them as RIF resistant.[34,35] Mutations detected by probe B of the Xpert® MTB/RIF are more likely to be associated with a silent/synonymous mutation than those mutations detected by other probes. Xpert® MTB/RIF detection of the *rpoB* silent mutation (Phe431Phe, formerly Phe514FPhe) as conferring RIF resistance has also been reported.[36] It has also been reported that the Xpert® MTB/RIF assay may generate occasional false-positive RIF resistance prediction for paucibacillary samples due to delays in the real-time signal generated by assay probes D and E.[37] Other methods such as DNA sequencing can identify these as silent mutations and negate the possibility of reporting of these mutations as conferring RIF resistance. For this reason, DNA sequencing is recommended to confirm probe-based detection of RIF resistance mutations. If this cannot be performed at the laboratory performing the assay, samples may be sent to the US Centers for Disease Control & Prevention's (CDC) Molecular Detection of Drug Resistance (MDDR) Program.

Low-level RIF Resistance

As previously described, one of the major challenges with RIF resistance testing is understanding the clinical impact of *rpoB* mutations associated with low-level RIF resistance. It is recognized that the frequency of such mutations varies by study.[13,15,28,38–40] Currently, efforts are ongoing to better characterize the frequency and impact of these mutations in the US.[15] It is important to note that these mutations can easily remain undetected using the current standard DST methods, including the rapid, broth based systems although there is an effort to correct this discordance by WHO as mentioned above.[8] Therefore, if only growth-based DST is used, some MTBC isolates that contain these mutations will be classified as RIF susceptible. Case reports in the literature suggest that treatment failure from RIF has been reported with isolates containing these types of mutations.[13,17] This has recently become an area of significant interest, and additional information about these mutations is rapidly becoming available. As more is learned, it could impact or alter the current DST methods and/or lead to the development of new approaches to identify RIF resistance more accurately.

To assure detection of RIF resistance, some laboratories have implemented testing RIF by broth microdilution to determine MIC, sequencing methods[28,41,42] or longer MGIT assay incubation times[43] which all enable detection of the presence of RIF resistance when low-level resistance caused by certain *rpoB* mutations are present.

Quality Assurance

CLIA certified laboratories must participate in a CLIA-approved proficiency testing (PT) program to satisfy regulatory requirements

for DST performance. If a qualified program is not available a suitable alternative must be implemented such as inter-laboratory comparison. In the US, the College of American Pathologists offers PT for RIF and other antituberculosis drugs; however, their program provides only two challenge isolates per year and includes only pan-susceptible strains. The Wisconsin State Laboratory of Hygiene Proficiency Testing also offers PT for molecular detection of MTBC and RIF resistance twice per year with five challenge samples per shipment. Other commercial PT programs, such as American Proficiency Institute, do provide PT challenges for MTBC DST. CDC offers the Model Performance Evaluation Program (MPEP) for MTBC DST, which is not a formal, graded PT program but could be used as an adjunct to the laboratory's regulatory PT program. MPEP is an educational self-assessment tool offering five MTBC isolates per challenge of which both drug resistant and drug susceptible isolates are included. It provides an opportunity to compare results to those obtained by other participants using the same methods.

IMPACT ON CLINICAL OUTCOMES

In 2020, 56 new MDR TB cases were reported in the US, 0.2% of cases among US born persons and 1.3% among non-US born persons.[44] RIF resistance is often found in combination with resistance to isoniazid (INH) and resistance to at least these two drugs is the definition of MDR TB. For this reason, mutations in the RNA polymerase β-subunit or *rpoB* gene (which confers RIF resistance) are often used as a surrogate for detecting MDR TB. Monoresistance to RIF has also been reported in the US at rates of <1%[45] and in other countries at rates between 7- 33%;[46,47] with recent reports suggesting that detection of RIF should no longer be used to predict MDR TB in some countries.[48]

The clinical impact of *rpoB* gene mutations associated with low-level RIF resistance on TB patients being treated in North America is limited[17] but an understanding of the prevalence and susceptibility of these mutations is beginning to be further understood.[8,15,49] Clinicians can be made aware of the presence of a *rpoB* gene mutation conferring low-level resistance if detection of the mutation by Xpert® MTB/RIF is followed by *rpoB* gene sequencing as recommended elsewhere in this document. Because RIF retains some *in vitro* activity in the presence of these mutations, clinicians have included a rifamycin in the treatment regimen, while not relying on RIF as they normally would and using an increased dosage.[50–52] The latest WHO guidelines indicate treatment with an MDR TB regimen is recommended for patients when mutations associated with low-level resistance are identified.[8,53]

In February 2022, CDC published interim recommendations for use of a four-month all-oral treatment regimen for drug-susceptible pulmonary TB in adults (older than 12 years).[54] These recommendations were based on results of a randomized clinical trial that compared the standard 6 month, currently recommended treatment regimen with a shorter regimen that included an intense phase consisting of daily INH, PZA, moxifloxacin, and high dose rifapentine for eight weeks, followed by nine weeks of daily INH, moxifloxacin and high-dose rifapentine.[55] Before initiating this regimen, rapid baseline molecular DST and growth-based DST are recommended, to include testing for INH, PZA, fluoroquinolones (moxifloxacin), and rifampin (proxy for rifapentine). Follow-up susceptibility testing also may be required to assess slow or non-responsiveness to treatment, e.g., if sputum cultures, collected at least monthly to culture conversion, remain positive at eight weeks. The availability of shorter oral regimens will enable patients to be cured faster, and has the potential to reduce treatment costs, improve patient quality of life and increase completion of therapy.

Rifabutin

Rifabutin (RFB) is a member of the rifamycin class of drugs and is recommended for TB treatment in RIF susceptible HIV co-infected patients on anti-retroviral therapy. Certain *rpoB* gene mutations are associated with a phenotype of RIF resistant, RFB-susceptible, especially mutations at codons 435 and 445 (formerly codons 516 and 526).[53] Some suggest that RFB DST should be performed when RIF resistance is observed.[57] Test concentrations for RFB should be 0.5 µg/mL in the MGIT assay or AP.[56] The clinical utility of RFB when such a mutation is found has not been established, however, RFB has been used in these situations based on its *in vitro* activity.[57,58]

It should be noted that certain mutations in the RRDR of the *rpoB* gene appear to confer cross-resistance to both RIF and RFB.[11,59–62] The mutations Ser450Leu, His445Tyr and His445Asp (formerly Ser531Leu, His526Tyr, and His526Asp) are most common and are found in isolates from a majority of MDR TB patients. Studies have demonstrated the association of various RRDR mutations with the differential expression of phenotypic resistance or susceptibility to RIF and RFB as measured by the MGIT assay.[63,64] These data may serve as a starting point to establish a database containing MICs of RFB and RIF associated with specific *rpoB* mutations, which will evolve as new mutations are detected and new MICs added.[63] Clinical studies to assess RFB treatment in certain MDR TB cases are needed.[63] MDR TB is often difficult to treat, therefore, development of a collection of evidence regarding the potential use of RFB for treatment of RIF resistant tuberculosis is desirable.

Rifapentine

Rifapentine (RPT), a derivative of RIF, has a longer half-life and a lower minimum inhibitory concentration for TB treatment than rifampin.[65] Rifapentine was approved by FDA in 1998 for treatment of pulmonary tuberculosis in combination with other antituberculosis drugs, administered twice weekly during the initial two months of treatment followed by once weekly for an additional four months. As treatment regimens have shifted towards shortening the overall duration of treatment, the potential for daily rifapentine dosing in shortened regimens was explored. Animal studies indicated that rifapentine, dosed daily had potent antimycobacterial activity that was associated with the ability to achieve cure without relapse after about three months of total treatment.[66] More recently a phase two clinical trial demonstrated a strong drug exposure-response effect for rifapentine using an intermediate marker of time to stable culture conversion,[67,68] an indicator of overall efficacy of an antituberculosis regimen. As described above, the clinical trial of the four-month treatment regimen including high-dose rifapentine and moxifloxacin has also shown a newer role for rifapentine in TB treatment.[55] To date studies on *rpoB* mutations associated with RIF resistance appear to also be associated with rifapentine resistance.[10,69]

AREAS OF ONGOING RESEARCH

There is a low prevalence of RIF resistance in the US and limited data available on the clinical implications of certain *rpoB* mutations means that there is disagreement about how to interpret these mutations and treat patients. It may be important to assess other published studies. Clinical impact of *rpoB* mutations in patients from Bangladesh or Africa could conceivably be different from impact on patients in North America, due to differences in nutritional status, disease severity at initiation of treatment, variations in drug quality, and/or differences in the quality of supervision of the directly observed therapy. Studies in North America may take longer to perform, because of a lower prevalence of RIF resistance. However, approximately 10% of *rpoB* mutations found in the US are associated with low-level resistance (personal communication, James Posey). Continued assessment of the frequency of occurrence of *rpoB* mutations associated with low-level RIF resistance in North America should be performed, to assess the cost-effectiveness of potential changes in the DST protocols for rifamycins.

A recent analysis concluded that molecular-based genotypic DST should be used to replace growth-based phenotypic DST results when mutations associated with low-level RIF resistance are identified due to a delayed growth in the MGIT assay.[43] However, additional research has shown that the MTBDR*plus* line probe assay could be used as a complementary test to confirm RIF DST results obtained using culture-based testing to rapidly assess the type of RIF mutations present.[70]

Additional Molecular Technologies for Identifying RIF Resistance

Novel molecular-based approaches to detect mutations associated with RIF resistance are being developed and are currently available outside of the US including the BD MAX™ MDR-TB (BD), the cobas® MTB-RIF/INH (Roche), Truenat MTB-RIF Dx assays (Molbio Diagnostics) and Xpert® MTB/RIF Ultra Assay (Cepheid). The BD MAX™ MDR-TB Panel and cobas® MTB-RIF/INH are both molecular assays for diagnosis of TB and detection of mutations associated with RIF and INH resistance. The Xpert® MTB/RIF Ultra assay (Ultra) and Truenat MTB-RIF Dx are both endorsed by the WHO but not currently available in the US. Initial evaluations of the Ultra assay indicate improved sensitivity and more definitive identification of RIF susceptibility and resistance.[29] Evaluations performed to date describe the changes in the Ultra results that may resolve discordance between a RIF resistant Ultra result and a low-level RIF susceptible phenotypic result due to a *rpoB* mutation conferring low-level RIF resistance.[16,71]

Sequencing Technologies to Identify Resistance

Another important area of ongoing research is the development of WGS[28,41,63,64] and tNGS[74–76] approaches that provide comprehensive analysis of the MTBC genome to more accurately assess genotypic DST. It has been suggested that sequencing of all mutations with a method such as WGS offers advantages over line-probe assays and other commercial molecular assays.[28,41,77,78] There is value in detecting all mutations as this can result in a higher sensitivity and provide more definitive data on which TB drugs to utilize, but these methods typically require cultured material and can take many days to test and report findings compared to currently available PCR and pyrosequencing methods. Molecular DST using WGS can be accomplished for additional and even novel drugs at no additional cost, contingent only on the knowledge base of characterized mutations and this testing data could be helpful when designing new treatment regimens.[41] Reports of shorter turnaround-times compared to growth-based DST with results available 7-8 days from a positive culture have also been described with the implementation of this testing.[28,77,79] Comprehensive WGS approaches and evaluation of data on low-level resistance mutations may continue to inform the best practices for treatment when strains harboring these mutations are present. tNGS enables the detection of a

significantly large enough portion of relevant mutations and could serve a similar role as WGS but the added benefit that may be applied directly to specimens, provide results more quickly than WGS, and at a lower cost.[65–67]

Studies describing the potential to perform tNGS directly from a specimen have been published[74,75,80] including a commercially available product, the Ion AmpliSeq™ TB panel (Life Technologies, Carlsbad, CA, US), that provides full-length gene analysis using ion semiconductor NGS.[80,81] Another RUO system, Deeplex® Myc-TB (Genoscreen, Lille, France), uses deep sequencing of a 24-plexed amplicon mix for simultaneous identification of mycobacterial species, genotyping, and prediction of drug resistance of MTBC. This includes 18 gene regions associated with resistance to 15 first- and second-line drugs.[82–85] The ability to provide comprehensive detection of mutations and derive additional information on the MTBC genome before culture is available could have a dramatic impact on TB diagnosis and patient management. Approaches for the direct sequencing of sputum are currently slower than rapid methods for detecting resistance but can assess susceptibility to a wider range of drugs and concurrently provide relatedness among MTBC strains, supporting infection control efforts to understand transmission.

Potential Changes to Standard Therapeutic Dose

Finally, studies and new data continue to emerge about possibly increasing the standard dose for treatment with RIF. If a change in treatment recommendation were to occur this could impact protocols for testing and reporting RIF by molecular and growth-based methods.[14,51,78] Additionally, if the recommended RIF dosage increases, the significance of low-level resistance could be impacted as well.

GUIDANCE

Laboratories should consider the following:

- Use of rapid detection of MTBC and RIF resistance directly on the clinical specimen, or alternative molecular method for screening for MDR TB, should be encouraged in all cases of TB-suspect patients. A molecular screening test should indicate when there is a mutation in the RRDR. DNA sequencing should be performed promptly to confirm resistance and identify if the mutation is a silent or low-level resistance mutation.
- Growth-based DST may be interpreted as susceptible for some strains of TB that have low-level resistance mutations detected by a molecular method. In these cases, laboratories should be available for consultation on test interpretation.
- Care must be taken to follow instructions and assure adequate vortexing of inoculum to break up clumps of MTBC and to allow sufficient time for settling of clumps in the suspension before inoculation of the drug tubes to avoid potential issues with false-positive or negative results.
- If your laboratory is unable to perform molecular testing for RIF, samples can be referred to a jurisdictional public health laboratory for testing and/or those laboratories can refer to the following services:
    - CDC Molecular Detection of Drug Resistance Service (MDDR) which provides rapid algorithm-based testing and other comprehensive testing services at no cost to all public health laboratories.
    - The National Public Health Laboratory Drug Susceptibility Testing (DST) Reference Center for *Mycobacterium tuberculosis* provides rapid algorithm-based testing at no cost to enrolled public health laboratories. Enrollment is restricted to public health laboratories performing TB DST on fewer than 50 isolates/year.
- When a *rpoB* mutation associated with low-level RIF resistance is found, the clinician(s) caring for the patient should be encouraged to seek expert consultation. Persons infected with strains with these mutations may be associated with diminished treatment success.

ABBREVIATIONS

AP	Agar proportion
CDC	US Centers for Diseases Control and Prevention
CFU	Colony forming units
CLIA	Clinical Laboratory Improvements Amendment
CLSI	Clinical and Laboratory Standards Institute
DST	Drug susceptibility testing
FDA	US Food and Drug Administration
INH	Isoniazid
LiPA	Line probe assay
LDT	Laboratory developed test
MDR TB	Multidrug-resistant tuberculosis
MGIT	*Mycobacterium* growth indicator tube
MIC	Minimum inhibitory concentration
MPEP	Model Performance Evaluation Program
MTBC	*Mycobacterium tuberculosis* complex
OADC	Oleic albumin dextrose catalase
PSQ	Pyrosequencing
PT	Proficiency testing
PZA	Pyrazinamide
RIF	Rifampin
RFB	Rifabutin
RPT	Rifapentine
RRDR	Rifampin resistance determining region
RUO	Research use only
TB	Tuberculosis
tNGS	Targeted next generation sequencing
WGS	Whole genome sequencing
WHO	World Health Organization

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