

**Mycobacteriology Reference Laboratory
Division of TB Elimination**

**Laboratory User Guide:
Molecular Detection of Drug Resistance
(MDDR)
in *Mycobacterium tuberculosis* Complex
by DNA Sequencing**

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Molecular Detection of Drug Resistance (MDDR) by DNA Sequencing

Introduction

What is the need for MDDR?

The ability to rapidly and accurately detect drug resistance in *Mycobacterium tuberculosis* clinical isolates is critical for the appropriate treatment of patients suffering from tuberculosis (TB) and the effectiveness of TB control programs. Efforts to treat patients and control the spread of tuberculosis can be hindered by the emergence of *M. tuberculosis* clinical isolates resistant to both first and second line anti-tuberculosis drugs. Additionally, the slow growth rate of *M. tuberculosis* and inherent difficulties associated with conventional drug susceptibility testing methods often serve as impediments to obtaining timely results. To address these issues, the Mycobacteriology Laboratory Branch (MLB) of the Division of Tuberculosis Elimination (DTBE) at the CDC is implementing a molecular testing service using DNA sequencing for the identification of drug resistance associated mutations in isolates of *M. tuberculosis* Complex (MTBC). This service (MDDR) will allow rapid confirmation of MDR TB through the identification of genetic mutations associated with rifampin (RIF) and isoniazid (INH) resistance. In addition, genetic loci associated with resistance to the most effective second-line drugs, fluoroquinolones (FQ) and the injectables amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) will be examined. All testing and reporting will be CLIA compliant.

http://www.cdc.gov/ncidod/srp/2008-2010_CLIA_Certificate_ocr.pdf

What is known about the genetic basis of resistance in *M. tuberculosis*?

The phenotypic drug resistance of clinical isolates of *M. tuberculosis* as determined by conventional methods (e.g, broth and agar proportion.) is explained by the presence of mutations in specific genes. These mutations often consist of only a single nucleotide change in the DNA sequence (i.e., point mutation). For example, >95% of clinical isolates that are resistant to RIF have a single point mutation in an 81-bp region of the *rpoB* gene known as the RIF resistance determining region (RRDR) (1). Mutations in this region affect binding of RIF to the target; thus, conferring resistance. Similarly, 70-90% of INH resistant isolates can be detected by sequencing the *inhA* promoter region, the *inhA* gene, and the *katG* gene (1). INH resistance can be attributed to mutations in the *inhA* promoter region which lead to overproduction of the drug target and mutations within *katG* which inhibit activation of the INH prodrug. Rapid detection of the presence of these mutations in *rpoB*, *inhA*, and *katG* can indicate that the isolate is resistant to RIF and/or INH weeks before conventional DST results would typically be available.

Though the genetic basis of resistance for some of the first and second line anti-tuberculosis drugs has been identified, some resistant isolates have unexplained mechanisms of resistance. As a result, the interpretation of molecular assays examining mutations associated with resistance must be done carefully and with a thorough understanding of the limitations of the test results. Although the presence of a mutation indicates that a clinical isolate is most likely resistant to the drug of interest; the absence of a mutation is **not** confirmation of drug susceptibility.

Molecular Detection of Drug Resistance (MDDR) service

What technology will be used?

Conventional PCR and DNA sequencing will be performed . Utilization of DNA sequencing technology was chosen as the platform for multiple reasons. First, the platform is semi-automated. In addition, the assay provides rapid results with extensive information regarding the specific mutations as well as some evidence of mixed populations of *M. tuberculosis*. Another benefit is the ease of expansion; as new mutations associated with resistance are defined, additional loci can be added to the sequencing panel relatively quickly.

What genetic loci will be sequenced as part of the MDDR service?

The sequencing panel was selected to be able to detect resistance associated mutations for the first- and second-line anti-tuberculosis drugs that define MDR- and XDR-TB.

(MDR-TB is defined as resistance to at least RIF and INH; XDR-TB is defined as MDR-TB plus resistance to a FQ and at least one of the second-line anti-TB injectable drugs: KAN, CAP or AMK.). Specific regions (loci) associated with genes previously reported to confer resistance will be sequenced including “hot spots” in *rpoB* (81 bp region associated with RIF resistance), *inhA* (promoter region) and *katG* (associated with INH resistance), *gyrA* (associated with resistance to FQs), *rrs* (associated with resistance to KAN, AMK, and CAP), *tlyA* (associated with CAP resistance), and *eis* (promoter region associated with KAN resistance). MDDR results will be issued in an interim report as soon as they are available. All isolates will also undergo conventional DST using agar proportion to determine phenotypic resistance to first- and second-line drugs (RIF, INH, ethambutol, streptomycin, ofloxacin, ciprofloxacin, KAN, CAP, AMK, ethionamide, and

Para-aminosalicylic). PZA testing will be performed by the MGIT 960 method.

Molecular and conventional results will be analyzed and released in a final report.

How was the MDDR service validated in the MLB at CDC?

Retrospective phase:

254 isolates from both US- and foreign-born patients referred to CDC for DST between the years 2000-2008 were selected for sequencing based on conventional DST results.

The collection included 163 (52%) MDR-TB, 10 (3%) XDR-TB, and 58 (18%) pan-susceptible isolates. If sequencing results differed from conventional DST, the conventional DST and sequencing was repeated, as needed, for confirmation.

Prospective phase:

85 consecutive isolates submitted June-August 2009 were simultaneously tested using MDDR and conventional methods to detect drug resistance. During this phase of validation, unidirectional workflow was established, , appropriate testing algorithms were developed, and QC and QA measures implemented. The specificity of MDDR for MTB complex was verified by completing the molecular assay on representative examples of nontuberculous mycobacteria species and members of the *M. tuberculosis* complex.

Performance:

Combined sensitivity and specificity for both the retrospective and prospective validation phases for MTB isolates were calculated using conventional drug susceptibility results (agar proportion method) as the gold standard.

Drug	Mutation	Sensitivity (%)	Specificity (%)
RIF	<i>rpoB</i>	96.1	97
INH	<i>inhA + katG</i>	88.6	98.7
FQ	<i>gyrA</i>	82.2	97
KAN	<i>rrs + eis</i>	86.8	96.9
AMK	<i>rrs</i>	87.9	99
CAP	<i>rrs + tlyA</i>	44.6	85.9

What are the expected limitations to MDDR?

The limitations of the molecular service can be attributed to gaps in knowledge. One limitation is that the clinical relevance of some mutations remains unknown. Many times sequencing will identify point mutations known to be associated with resistance to a particular drug; however, sometimes the association of detected mutations with resistance will be unknown due to insufficient genetic data. An additional limitation is that not all mechanisms of resistance are known. Therefore, if no mutation is detected by the

molecular assay, resistance can not be ruled out. Conventional DST results are essential to confirm susceptibility to individual drugs.

Use of the MDDR service by submitters

What are the MDDR submission criteria for MTBC clinical isolates?

Isolates may be submitted for molecular detection of drug resistance if one of the following criteria is met:

- 1) High-risk of RIF resistance or MDR-TB (including previously treated TB case, drug resistant TB contact, foreign-born from area with high rates of MDR TB)
- 2) Known RIF resistant isolates
- 3) High profile patients (e.g. daycare workers, nurses)
- 4) Adverse reactions (e.g. patient allergic to RIF)
- 5) Mixed or non-viable cultures
- 6) other situations considered on case by case basis

What sample types will be accepted?

Isolates of *M. tuberculosis* complex will be accepted for the service. MDDR testing has not been validated for clinical specimens (e.g., raw or processed sputum). Isolates can be submitted on either solid media (e.g. LJ or Middlebrook) or as positive MGIT cultures. Validation of MDDR testing from other types of liquid media is underway and may be accepted in the future. Bactec460 bottles will not be accepted. The laboratories should submit only one sample per patient; however, duplicate samples can be considered on a case by case basis.

In order to effectively utilize rapid methods, please submit eligible culture isolates as soon as they are identified as MtbC. In most cases, a positive MGIT culture will be available before a solid culture. When shipping liquid culture, please send 1 ml of culture in a screw-cap cryovial that has been sealed with parafilm.

How do I submit an isolate for the MDDR service?

Submitters should complete the MDDR request form and submit via email to TBLab@cdc.gov. Once approved, MLB will send an email with further submission instructions and a DASH form. Please attach the MDDR request form to the DASH form when shipping isolates. Isolates should be shipped via overnight service to CDC Monday through Thursday only. Do not ship isolates on Friday.

http://www.cdc.gov/ncidod/dvbid/misc/CDC50_34.pdf (DASH form)

<http://www.cdc.gov/ncidod/srp/specimens/shipping-packing.html> (shipping information)

How will MDDR results be reported?

An interim report will be issued to the submitter when MDDR results are available. This report will contain appropriate patient identification and submitter information. Results will be summarized in a table to indicate the locus sequenced, sequencing results (WT or amino acid change designation), and interpretation of sequencing results in terms of what is known about the association of the mutation with drug resistance.

A final report will be issued when conventional DST results are available. This report will contain both the molecular and conventional DST results and final drug susceptibility profile. Comments regarding discordance, if appropriate, will be included.

References

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- 2) C. Maus et al. *AAC*. 49(8):3192-3197, 2005.
- 3) S. Feuerriegel et al. *AAC*. Epub, 2009.
- 4) M. Zaunbrecher et al., submitted