

**Reference Laboratory
Division of TB Elimination**

**Laboratory User Guide
for U.S. Public Health Laboratories:
Molecular Detection of Drug Resistance
(MDDR)
in *Mycobacterium tuberculosis* Complex
by DNA Sequencing (Version 2.0)**

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Molecular Detection of Drug Resistance (MDDR) by DNA Sequencing (Version 2.0)

Introduction

What is the advantage of MDDR?

The ability to rapidly and accurately detect drug resistance in *Mycobacterium tuberculosis* Complex (MTBC) is critical for the effective treatment of patients suffering from tuberculosis (TB) and relevant interventions of TB control programs. Efforts to treat patients and control the spread of TB can be hindered by the emergence of MTBC resistant to both first and second line anti-TB drugs. Additionally, the slow growth rate of MTBC and inherent difficulties associated with conventional drug susceptibility testing methods often serve as impediments to obtaining timely results. Since September 2009, the Laboratory Branch of the Division of Tuberculosis Elimination at U.S. CDC has offered a molecular testing service using conventional DNA sequencing for the identification of drug resistance associated mutations in isolates of MTBC. In June 2012, the service was expanded by incorporating pyrosequencing (PSQ) into the testing algorithm and by accepting nucleic acid amplification-positive (NAAT+) sputum sediments for testing to provide the ability for local providers and programs to potentially further reduce delayed diagnosis of MDR TB. The service allows rapid identification of multidrug-resistant (MDR) TB through the detection of genetic mutations associated with rifampin (RMP) and isoniazid (INH) resistance. In addition, when resistance to RMP is already known or detected in the MDDR service, genetic loci associated with resistance to ethambutol (EMB), pyrazinamide (PZA), and the most effective second-line drugs, fluoroquinolones (FQ) and the injectables amikacin (AMK), kanamycin (KAN), and capreomycin (CAP), are examined.

Testing and reporting is CLIA compliant.

<http://www.cdc.gov/ncezid/pdf/cli-atl-other-2011-2013.pdf>

What is known about the genetic basis of resistance in MTBC?

The phenotypic drug resistance of clinical isolates of MTBC as determined by conventional, growth-based, drug susceptibility testing (DST) methods 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 RMP have a single point mutation in an 81-bp region of the *rpoB* gene known as the RMP resistance determining region (RRDR) (1). Mutations in this region affect the protein structure of the target so that RMP cannot bind; 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 RMP 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-TB drugs has been identified (2,3), some resistant isolates have unexplained mechanisms of resistance. As a result, the interpretation of molecular assays examining mutations associated with resistance must be done with a thorough understanding of the limitations of the test results. Although the presence of many mutations 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.

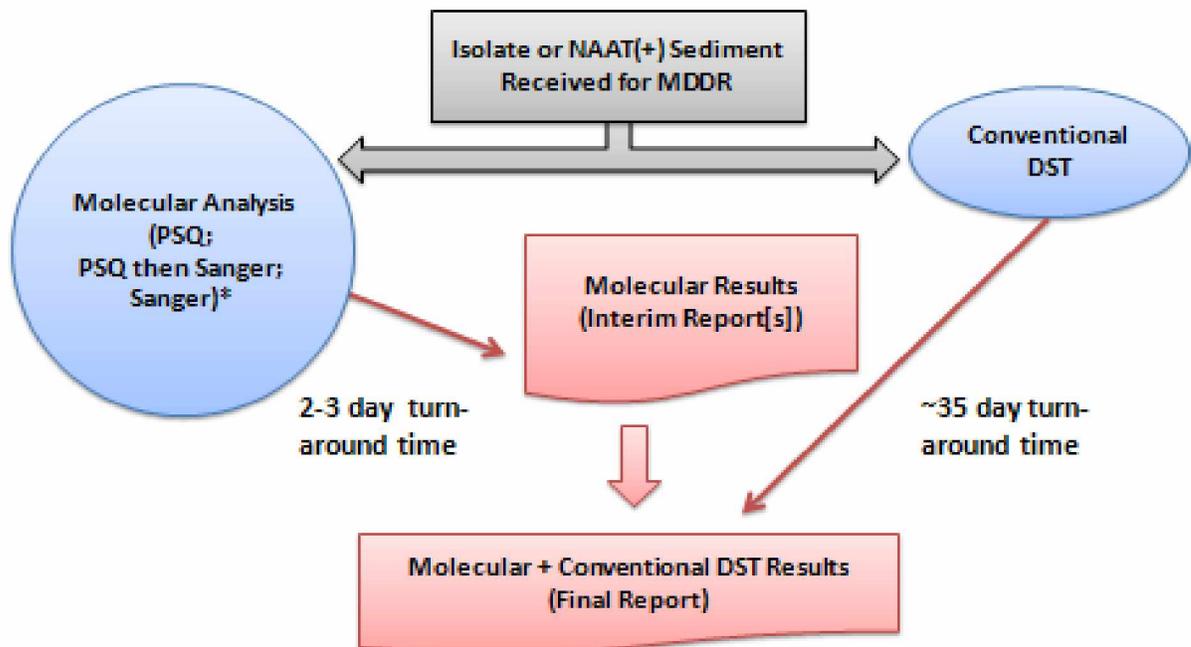
Molecular Detection of Drug Resistance (MDDR) service

What technology will be used?

DNA sequencing technology was chosen as the platform for MDDR for multiple reasons. First, the platform is semi-automated. In addition, the assay provides rapid results with

extensive information regarding the specific mutations. Conventional PCR and pyrosequencing (PSQ) will be performed on submitted sputum specimen sediments where initial nucleic acid amplification (NAA) testing for MTBC is positive and on submitted isolates of MTBC when the RMP susceptibility results are unknown (e.g., testing not completed or in progress); this testing will include only genetic loci associated with INH and RMP resistance. When a mutation is detected by PSQ that indicates possible RMP resistance, testing will automatically reflex to conventional (i.e., Sanger) DNA sequencing of the comprehensive panel (genetic loci associated with resistance to INH, RMP, EMB, PZA, FQ, and second-line injectable drugs). When an isolate of MTBC or NAAT+ sputum sediment is submitted with known resistance to RMP, or when clinical indications require a more comprehensive testing panel, PSQ will be bypassed and the comprehensive panel testing will be performed. The testing algorithm used for each sample submitted will be determined by CDC based on information on the MDDR request form.

MDDR V2.0 Algorithm



*based on information supplied on request form

Sediments will be inoculated into growth medium to obtain an MTBC isolate. All isolates, including those isolated from sputum sediments, will also undergo conventional DST using the agar proportion method to determine phenotypic resistance to first- and second-line drugs (RMP, INH, EMB, streptomycin, ofloxacin, ciprofloxacin, KAN, CAP, AMK, ethionamide, and para-aminosalicylic). PZA testing will be performed by the MGIT 960 method.

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

The PSQ panel and the comprehensive panel were designed to be able to detect resistance associated mutations defining MDR TB and extensively drug-resistant (XDR) TB, respectively. (MDR TB is defined as resistance to at least RMP 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 RMP resistance), *inhA* promoter region and *katG* (associated with INH resistance), *embB* (associated with EMB resistance), *pncA* (associated with PZA resistance), *gyrA* (associated with FQ resistance), *rrs* (associated with resistance to KAN, AMK, and CAP), *tlyA* (associated with CAP resistance), and *eis* (promoter region associated with KAN resistance).

What are the performance characteristics of MDDR at CDC?

Combined sensitivity and specificity for the retrospective (254 clinical isolates) and prospective (80 clinical isolates) validation phases for MDDR as well as the results of 225 clinical isolates submitted for MDDR analysis (Sept2009—Feb2011) were calculated using conventional drug susceptibility results (agar proportion method) as the gold standard. For PZA, sensitivity and specificity were calculated using MGIT PZA results as the gold standard. Presence or absence of a mutation in *pncA* was used to indicate resistance and susceptibility by MDDR, respectively. However, the clinical significance of many of the mutations detected in *pncA*, with respect to prediction of PZA resistance, is unknown.

Performance characteristics of MDDR by Drug			
Drug	Locus or loci examined	Sensitivity (%)	Specificity (%)
RMP	<i>rpoB</i>	97.1	97.4
INH	<i>inhA + katG</i>	86.0	99.1
FQ	<i>gyrA</i>	79.0	99.6
KAN	<i>rrs + eis</i>	86.7	99.6
AMK	<i>rrs</i>	90.9	98.4
CAP	<i>rrs + tlyA</i>	55.2	91.0
EMB	<i>embB</i>	78.8	94.3
PZA	<i>pncA</i>	86.0	95.9

What are the limitations to the MDDR service?

The limitations of the MDDR service can be attributed primarily to gaps in knowledge and the limit of detection of the assays being used. One limitation is that the clinical relevance of some mutations remains unknown. Many times sequencing will identify mutations known to be associated with resistance to a particular drug; however, sometimes the association of detected mutations with resistance will be unknown because the mutation is novel or there is insufficient genetic data to definitively associate the mutation with resistance. 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. Genetic analysis may miss mutations in heterogeneous samples (i.e., only a portion of cells in the population being tested carry a mutation associated with resistance).

Used alone, MDDR and conventional DST are imperfect, yet when used in conjunction with one another, accuracy of the detection of drug resistance can be improved. Conventional DST results are still essential to confirm susceptibility to individual drugs. However, there are situations when MDDR results indicate resistance due to the presence of a mutation yet the conventional DST results indicate susceptibility. For example,

certain mutations in *rpoB* are associated with RMP resistance but drug susceptibility testing may be falsely susceptible (4). Ongoing studies at CDC and other laboratories will inform our understanding of these situations.

Use of the MDDR service by submitters

What are the submission criteria for MDDR?

Isolates of MTBC and NAAT+ positive processed sputum sediment specimens may be submitted by U.S. Public Health Laboratories for MDDR if one of the following criteria is met:

- 1) By patient history, there is a high-risk of RMP resistance or MDR TB (including those previously treated for TB, a contact of drug resistant TB, who are foreign-born from an area with high rates of MDR TB)
- 2) Known RMP resistance (by rapid test or by culture-based DST)
- 3) Patients where the result of drug resistance will predictably have a high public health impact (e.g., daycare workers, nurses)
- 4) Patient is known to have certain adverse reactions to critical anti-TB drug (e.g., allergy to RMP)
- 5) Mixed or non-viable cultures
- 6) Isolates which fail to grow in DST medium
- 7) Other situations considered on case by case basis

What sample types will be accepted?

NAAT(+) sputum sediments and isolates of MTBC will be accepted. Isolates can be submitted on either solid media (e.g., LJ or Middlebrook) or as broth cultures. When shipping sputum sediment or broth culture, please send 0.5—1 ml in a screw-cap cryovial that has been sealed with parafilm. Do not send any samples in 50 ml conical tubes. Bactec460 bottles will not be accepted. Only one sample per patient should be submitted; however, testing of duplicate samples will be considered on a case by case basis.

MDDR does not take the place of conventional culture and DST for MTBC. Standard of care testing (i.e., smear, culture, and DST) should be performed in the submitting laboratory when NAAT(+) sputum sediments are submitted for MDDR testing.

How do I submit a sample for the MDDR service?

Submitters should complete all portions of the MDDR request form and submit via email (TBLab@cdc.gov) or FAX (404-639-5491). Once approved, CDC will send an email with further submission instructions. Please attach the MDDR request form to the CDC requisition (http://www.cdc.gov/ncidod/dvbid/misc/CDC50_34.pdf) when shipping. Sediments and isolates should be shipped via overnight service to CDC Monday through Thursday. Do not ship on Friday. Liquid samples do not need to be shipped on dry ice.

Samples should be shipped in compliance with federal regulation.

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

Shipping address:

ATTN: STAT Lab
B. Metchock, Unit 29
1600 Clifton Road, NE
Atlanta, Ga. 30333
770-488-7100

Questions regarding the use of the APHL's FedEx Account for shipment should be addressed to APHL (tam.van@aphl.org; kelly.wroblewski@aphl.org).

How will MDDR results be reported?

Reports will be issued by FAX to the submitting laboratory when the PSQ panel or the comprehensive panel results are available. A final report will be issued when conventional DST results are available. Comments regarding discordance between conventional DST results and MDDR, if appropriate, will be included. The submitting laboratory is responsible for dissemination of CDC reports to the TB program / clinician as appropriate.

What if I have trouble interpreting MDDR results?

Laboratorians, TB control personnel, and clinicians can contact the CDC for help in interpretation of reports.

e-mail: TBLab@cdc.gov

Telephone: 404-639-2455

References

- 1) S.Ramaswamy and J.M. Musser. *Tubercle and Lung Disease*. 79(1):3-29, 1998.
- 2) S. Feuerriegel et al. *Antimicrob Agents Chemother*. 53(8):3353-3356, 2009.
- 3) Campbell et al. *Antimicrob Agents Chemother*. 55(5):2032-2041,2011
- 4) Van Duen et al. *J Clin Microbiol*. 47(11):3501-3506, 2009