# Characterization of Multi-drug Resistant *Mycobacterium tuberculosis* from Immigrants Residing in the United States using Next Generation Sequencing

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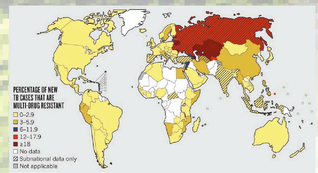
## Abstract

**Objective:** Drug-resistant *Mycobacterium tuberculosis* (MTB) is spreading worldwide. While this is a global threat, there is a growing concern in this country as the majority of MTB cases are from foreign-born persons currently residing in the United States. The use of Next Generation Sequencing (NGS) can be a practical tool to enhance early intervention and potentially provide a more meaningful characterization of multi-drug resistant (MDR) TB in the local community.

**Study Design:** The City of Milwaukee Health Department Laboratory isolated three drug-resistant strains from Burmese, Hmong, and Indian immigrants residing in Milwaukee, WI. Ion Torrent full-gene sequencing and complete genetic analysis was performed within five days of the receipt of isolates. Genotypic results obtained by NGS were compared to phenotypic results obtained by classical drug sensitivity testing (DST).

**Results:** Genetic characterization of seven, full-length resistance-associated genes revealed two MDRs and one highly resistant strain with important drug-resistant mutations that were subsequently confirmed by traditional DST. NGS revealed a novel Lys96Arg (K96R) *pncA* gene substitution mutation in one isolate that had not been formerly reported.

**Conclusions:** The rapid turnaround time from sample-to-sequence underscores the public health value of using an NGS approach for full-gene analysis of MDR cases from epidemiologically significant clinical isolates. This novel, non-culture based approach could be used for routine MTB drug resistance characterization as well as for potential discovery of new resistance-associated mutations, and would allow for improved understanding and characterization of drug-resistant mycobacteria.

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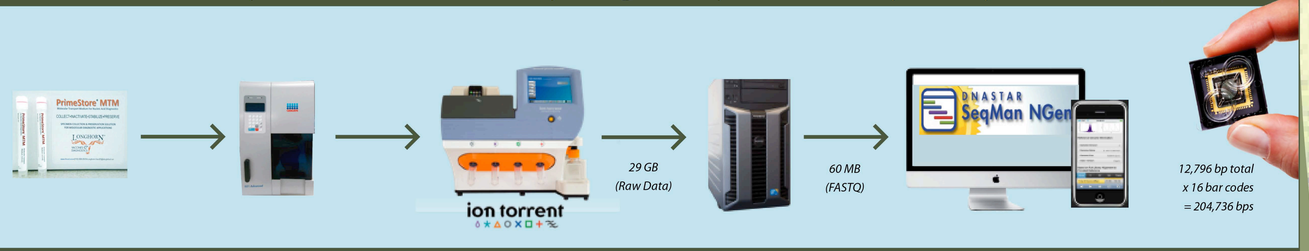
## From Receipt of Isolates and Full Gene Sequencing to Complete Genetic Analysis: 5 Days




TB Clinical Isolates | Extraction on Qiagen EZ1 | Next Gen Sequencing on PGM Ion Torrent | Bioinformatics using DNAStar

## Introduction

Increasing cases of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (MTB) continue to emerge, particularly from Asia and Africa[1]. In the United States, the majority of reported MTB cases have occurred in foreign-born persons[2]. MDR strains are resistant to rifampin and isoniazid, while XDR strains are resistant to rifampin and isoniazid plus one fluoroquinolone and one of the injectable drugs – amikacin, kanamycin, or capreomycin. However, some MDR strains have additional drug resistance, *i.e.* pyrazinamide and/or streptomycin that might decrease treatment efficiency. Recently, an Ion Torrent (Life Technologies, Carlsbad, CA) full-gene sequencing approach was developed for rapid characterization of MTB resistance mutations[3] that can be performed for clinical isolates within days.

## Specific Aim

Between April 2009 and November 2011, sputum specimens from three patients (Burmese, Hmong, and Indian immigrants residing in Milwaukee, WI) yielded antibiotic resistant strains of MTB. Antibiotic resistance profiles were deduced by full-length gene sequencing of three clinical isolates (two MDRs and one unique drug-resistant strain) on the Ion Torrent Next Generation Sequencing (NGS) platform. Sequencing data was compared to phenotypic data obtained by conventional drug sensitivity testing (DST).

## Methods

1. Sputum samples were confirmed for *M. tuberculosis* at the MHD laboratory using smear microscopy, MTB specific real-time PCR[4], culture and DST for phenotypic resistance using the BACTEC™ MGIT™ system (BD Diagnostic, USA)[5] with isoniazid (0-2 and 1-0 µg/ml), rifampin (1-0 µg/ml), and pyrazinamide (100 µg/ml).
2. H37Rv strain was used as an internal sequencing control. The MTB colonies grown on solid Löwenstein–Jensen medium was inactivated and transported in PrimeStore Molecular Transport Medium (MTM; Longhorn Vaccines & Diagnostics, USA) at ambient temperature for sequencing.
3. Total DNA was purified from 200 µl aliquots of PrimeStore MTM. PCR amplification using full-gene primers for *rpoB, katG, pncA, gyrA,* and *rrs* was performed[3]. Additional primers for full-gene amplification of *rpsL* and *inhA* were used for streptomycin and isoniazid resistance. PCR amplicons from clinical isolates were pooled, sheared, size-selected, and subjected to adaptor/barcoding ligation[3].
4. Ion Torrent sequencing was performed using 314 V2 chips with 200-bp sequencing chemistry (Life Technologies, Carlsbad, CA). Sequence assembly, alignments, and protein translations were performed using SeqMan NGen (V4) and LaserGene (V10) Core Suite (DNAStar, USA).

## Results

PCR was performed using seven novel primer pairs for full-length amplification of *rpoB, katG, inhA, gyrA, pncA, rpsL,* and *rrs* genes associated with MDR and XDR resistance in MTB *(Figure 2)*.

Full-length *rpoB, katG, inhA, pncA, rpsL, gyrA,* and *rrs* genes sequences showed 100% concordance with phenotypic DST through direct identification of amino-acid substitutions known to confer antibiotic resistance *(Table 1)*. All three cases were resistant to isoniazid at both low and high levels (0.1 µg/ml and 0.4 µg/ml) by phenotypic testing *(Table 1)*.

Cases 1 and 3 contained a characteristic Ser531Leu mutation associated with rifampin resistance[6], while case 2 contained the wild type residue. NGS revealed that cases 2 and 3 exhibited the common Ser315Thr katG mutation known to confer between 75% and 94% of isoniazid resistance worldwide[7]. Case 1 did not contain any *katG* gene mutations, but exhibited a much rarer Iso21Thr (I-21-T) substitution in the *inhA* gene that was previously correlated with isoniazid resistance, and a K96R pyrazinamide gene mutation *(Table 2)*.

**Table 1.** *Comparison of genotypic and phenotypic drug resistance* Ion Torrent sequencing† and drug sensitivity testing*

| | Case 1 | | Case 2 | | Case 3 | |
|---|---|---|---|---|---|---|
| | Ion Torrent | Bactec MGIT 960 | Ion Torrent | Bactec MGIT 960 | Ion Torrent | Bactec MGIT 960 |
| **Rifampin** | Resistant | Resistant | Sensitive | Sensitive | Resistant | Resistant |
| **Isoniazid** | Resistant | Resistant | Resistant | Resistant | Resistant | Resistant |
| **Pyrazinamide** | Resistant | Resistant | Sensitive | Sensitive | Sensitive | Sensitive |
| **Fluoroquinolone** | Sensitive | Sensitive | Sensitive | Sensitive | Sensitive | Sensitive |
| **Streptomycin** | Resistant | Resistant | Resistant | Partial resistance§ | Sensitive | Sensitive |
| *Resistance type* | *Multidrug resistant* | | *Drug resistant* | | *Multidrug resistant* | |

* Critical concentrations for indicated antibiotics are: rifampin (2-0 µg/ml); isoniazid (0-1 µg/ml); pyrazinamide (100 µg/ml); fluoroquinolone (2 µg/ml); streptomycin (2-0 µg/ml and 6-0 µg/ml).
† Previously confirmed amino-acid substitutions giving rise to antibiotic resistance.
§ Resistant at 2-0 µg/ml but sensitive at 6-0 µg/ml.

***Figure 2.*** Representative amplicons for 4 MTB genes (*rpoB, katG, gyrA,* and *rrs*). Seven genes were sequenced for each clinical isolate.

**Table 2.** *Amino-acid substitutions in drug resistance genes from* M. tuberculosis *samples isolated from Burmese, Hmong, and Indian immigrants residing in the USA using Ion Torrent sequencing**

| | Amino-acid substitution(s)[1] in full-length genes (antibiotic)[3] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Isolate no. | *rpoB* (rifampin) | *katG* (isoniazid) | *inhA* (isoniazid) | *pncA* (pyrazinamide) | *gyrA* (fluoroquinolones) | *rpsL* (streptomycin) | *rrs (165)* (aminoglycosides/ streptomycin) |
| **Case 1** | **S531L** | R463L | **121 T** | **K96R** | S95 T, E21Q, G688D | **K43R** | WT |
| **Case 2** | WT | **S315T**, R463L | WT | WT | WT | **K43R** | WT |
| **Case 3** | **S531L** | **S315T,** R463L | WT | WT | WT | WT | WT |

WT refers to the 'wild type' H37Rv reference strain.
Amino-acid changes in bold indicate a known resistance mutation.
* Compared to previously confirmed amino-acid substitutions know to confer antibiotic resistance.
† Compared to H37Rv wild-type reference strain.
§ Gene sequences: Case 1 (TB11-91), Case 2 (TB11-111), and Case 3 (TB11-153) were deposited in GenBank (accession numbers KC692347–KC692364).

SHORT REPORT
Characterization of multi-drug resistant Mycobacterium tuberculosis from immigrants residing in the USA using Ion Torrent full-gene sequencing

Correspondence

## Current Practices

*Phenotypic Drug Resistance Testing (DST):* Bactec MGIT (320/960) (BD Diagnostics, MD)- BACTEC PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin) for growth- Streptomycin (STR), Isoniazid (INH), Rifampin (RIF), Ethambutol (EMB) (collectively known as SIRE), and Pyrazinamide (PZA)

*Molecular Detection of Drug Resistance:*

1. GeneXpert TB/RIF assay for MTB and Rifampin (rpoB mutations)
2. Molecular Detection of Drug Resistance (MDDR) for rapid identification of MDR TB-DNA sequencing (PSQ) for detection of mutations (rifampin and isoniazid), followed by Sanger sequencing of comprehensive panel (resistance to INH, RMP, EMB, PZA, FQ, and second-line injectable drugs) at CDC

## Challenges

1. Phenotypic drug susceptibility testing (DST)- growth characteristics of MTB make it difficult to establish resistance (*e.g.* PZA)
2. Limited availability of commercial NAAT assays for MTB drug resistance
3. Inability to detect novel or unusual mutations

## Advantage of NGS

1. Improved TAT, workflow, consistency, and potential for library creation
2. Depth of coverage and characterization of mixed strain populations allows detection of hetero-resistance in populations (both resistant and susceptible subpopulations)
3. Potential for discovery of new mutations that may be associated with resistance
4. No laser detection or light-sensitive chemistries require fluorescent-labelled moieties
5. A small working footprint

## Limitations

1. Initial DNA concentration AND no direct amplification/library prep from primary sputum
2. Read-length varies for platforms
3. Lack of phenotypic Minimal Inhibitory Concentration (MIC) study for new resistance
4. Understanding type of amino-acid substitution- important for phenotypic DST Vs. NGS
5. Bioinfomatic capability limited

## Conclusions

- Global travel and mobility of pathogens will continue to increase the spread of TB and other communicable diseases.
- Drug-resistant TB continues to emerge globally; however, characterization of these strains remains incomplete.
- Improved and more rapid understanding of TB resistance-associated genes should decrease inappropriate treatment and transmission of MDR-TB globally.

**References**

[1] Global Report on Surveillance and Response. Geneva: WHO, WHO/HTM/TB/2010.3, 2010
[2] Centers for Diseases Control and Prevention http://www.cdc.gov/tb/statistics/reports/2011/pdf/report2011.pdf. 2013
[3] Daum LT, *et al.* 2012. J. Clin. Microbiol.50: 3831–3837
[4] Desjardin LE, *et al.* 1998. 36: 1964–1968
[5] Alcaide R, *et al.* 2009. J. Clin. Microbiol.38: 398–340
[6] Daum LT *et al.* 2013. Epidemiol. Infect. 27:1–6
[7] Daum LT *et al.* 2014. Clin Infect Dis. 58(6):903–4

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