JCM
Journals.ASM.org

Next-Generation Ion Torrent Sequencing of Drug Resistance Mutations in *Mycobacterium tuberculosis* Strains

Luke T. Daum,[a] John D. Rodriguez,[a] Sue A. Worthy,[a] Nazir A. Ismail,[b,c] Shaheed V. Omar,[b,c] Andries W. Dreyer,[b] P. Bernard Fourie,[b] Anwar A. Hoosen,[b] James P. Chambers,[a,d] and Gerald W. Fischer[a]

Longhorn Vaccines & Diagnostics, San Antonio, Texas, USA[a]; Department of Medical Microbiology, University of Pretoria, Pretoria, South Africa[b]; Centre for Tuberculosis, National Institute of Communicable Diseases (NICD), Sandringham, South Africa[c]; and University of Texas at San Antonio, San Antonio, Texas, USA[d]

A novel protocol for full-length *Mycobacterium tuberculosis* gene analysis of first- and second-line drug resistance was developed using the Ion Torrent Personal Genome Machine (PGM). Five genes—*rpoB* (rifampin), *katG* (isoniazid), *pncA* (pyrazinamide), *gyrA* (ofloxacin/fluoroquinolone), and *rrs* (aminoglycosides)—were amplified and sequenced, and results were compared to those obtained by genotypic Hain line probe assay (LPA) and phenotypic Bactec MGIT 960 analysis using 26 geographically diverse South African clinical isolates collected between July and November 2011. Ion Torrent sequencing exhibited 100% (26/26) concordance to phenotypic resistance obtained by MGIT 960 culture and genotypic *rpoB* and *katG* results by LPA. In several rifampin-resistant isolates, Ion Torrent sequencing revealed uncommon substitutions (H526R and D516G) that did not have a defined mutation by LPA. Importantly, previously uncharacterized mutations in *rpoB* (V194I), *rrs* (G878A), and *pncA* (Q122Stop) genes were observed. Ion Torrent sequencing may facilitate tracking and monitoring geographically diverse multidrug-resistant and extensively drug-resistant strains and could potentially be integrated into selected regional and reference settings throughout Africa, India, and China.

Mycobacterium tuberculosis, the causative agent for tuberculosis (TB), is a highly transmissible bacterial pathogen with significant morbidity and mortality, particularly in HIV-infected patients. Since 1997, tuberculosis has remained the leading cause of death in South Africa (19, 29), a statistic linked to this country's growing HIV epidemic (25). Moreover, effective treatment measures in patients with active *M. tuberculosis* have been hindered by increasing cases of multidrug-resistant (MDR) and extensively drug-resistant (XDR) clinical isolates.

MDR *M. tuberculosis* strains are resistant to first-line antibiotics rifampin (RIF) and isoniazid (INH), while XDR *M. tuberculosis* strains are resistant to both RIF and INH as well as fluoroquinolones (FQs) and second-line injectable antibiotic drugs, i.e., amikacin (AMK), kanamycin (KAN), and capreomycin (CAP). Approximately 9.6% of all TB cases are caused by MDR strains, and South Africa continues to report higher percentages of XDR cases each year (20, 28). There are approximately 10,000 newly reported cases of MDR TB each year in South Africa (30), with a mortality rate of more than 10% in patients infected with MDR *M. tuberculosis* strains (28). The emergence of antibiotic-resistant *M. tuberculosis* strains underscores the immediate need for rapid and highly accurate diagnosis, particularly in the developing countries of Africa.

Currently, the "gold standard" for identification of MDR strains is culture-based drug susceptibility testing (DST). However, it is time-consuming (weeks to months), technically challenging, and cost prohibitive, especially in resource-limited countries. For example, the Bactec MGIT 960 system (Becton Dickinson, Franklin Lakes, NJ) is an automated, continuously culture-based monitoring platform that measures bacterial oxygen consumption and is used for phenotypic DST. Drug susceptibility tests obtained using the Bactec MGIT 960 system are reliable and reproducible (1, 14); however, this methodology requires handling of viable and potentially infectious cultures, days to weeks until results are obtained, specialized laboratory facilities, expensive consumables, and instrument maintenance.

In recent years, several commercially available nucleic acid-based assays have been developed for determining *M. tuberculosis* drug resistance and have been approved for use by the World Health Organization (WHO). The Hain LifeScience Genotype MTBDR*plus* line probe assay (LPA) is one of the most widely used. This test entails nucleic acid extraction, PCR amplification, probe hybridization, and colorimetric visualization on lateral flow strips via an alkaline phosphatase-linked reaction. The GenoType MTBDR*plus* method cannot detect XDR *M. tuberculosis* strains and is limited to MDR strains via characterization of mutations in (i) *rpoB* gene (D516V, H526Y/D, and S531L), (ii) *katG* gene (S315T), and (iii) *inhA* promoters. These well-characterized mutations confer resistance to the antibiotics rifampin and isoniazid, respectively. The LPA has been shown to be both sensitive and specific using clinical isolates and/or smear-positive samples (6), but not without several drawbacks. The sensitivity of LPA for all resistance-associated mutations will realistically never reach 100%, since many mutations that confer resistance have yet to be discovered. Additionally, the LPA will not directly detect an amino acid substitution mutation outside a common and characterized mutation site or within a known resistance site but where the amino acid substitution represents an uncommon residue. Furthermore, an undefined mutation indicated by the absence of a wild-type band in LPA may not be phenotypically resistant.

Received 18 July 2012 **Returned for modification** 9 August 2012
Accepted 5 September 2012
Published ahead of print 12 September 2012
Address correspondence to Luke T. Daum, LTD@lhnvd.com.
Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JCM.01893-12

TABLE 1 PCR amplification primers used for full-length Ion Torrent analysis of *M. tuberculosis* genes

Amplification target	Forward primer	Reverse primer	Amplicon length (bp)
rpoB	5′-TCCTCTAAGGGCTCTCGTT-3′ (19 nt[a])	5′-GTCAGGTACACGATCTCGT-3′ (19 nt)	1,625
rpoBII (second half)	5′-ATCGAAACGCCGTACCGCAA-3′ (20 nt)	5′-TGACGTCGAGCACGTAACTCCCT-3′ (23 nt)	2,056
katG	5′-ACACCAACTCCTGGGAAGGAAT-3′ (21 nt)	5′-TGATCGCACATCCAGCACATTT-3′ (22 nt)	2,447
pncA	5′-GACGGATTTGTCGCTCACTAC-3′ (21 nt)	5′-GCCGGAGACGATATCCAGAT-3′ (20 nt)	960
gyrA	5′-AAGGATGTTCGGTTCCTGGAT-3′ (21 nt)	5′-TAACACTCGTACCCGGCT-3′ (18 nt)	2,664
rrs (16S)	5′-TTCTAAATACCTTTGGCTCCCT-3′ (22 nt)	5′-TGGCCAACTTTGTTGTCATGCA-3′ (22 nt)	1,680
Total			11,432 total (5 genes)

[a] nt, nucleotides.

The Ion Torrent is a next-generation semiconductor sequencing platform that utilizes a small chip for detection of released hydrogen ions emitted during DNA polymerization. The Ion Torrent instrument was first marketed in February 2010 by Life Technologies Incorporated (Foster City, CA) and is capable of sequencing megabases of raw sequence reads on a single chip.

In this report, we describe an Ion Torrent Personal Genome Machine (PGM) protocol for rapid (2 days), standardized, and cost-effective full-length gene analysis of *M. tuberculosis* genes. The developed protocol was used to assess first- and second-line *M. tuberculosis* drug resistance in 26 geographically diverse MDR and XDR clinical isolates collected between July and November 2011 from South Africa. Ion Torrent sequencing data were compared to data obtained by the Hain LPA and/or phenotypic DST using the Bactec MGIT 960.

MATERIALS AND METHODS

Clinical isolates. A random sampling of 26 drug-susceptible, MDR, and XDR clinical isolates (collected between July and November 2011) was selected from clinical isolates from the Gauteng and Kwa-Zulu Natal provinces of South Africa. The clinical specimens were initially confirmed by conventional culture methodology. The H37Rv *M. tuberculosis* lab strain was included as a sequencing control throughout the protocol. All *M. tuberculosis* isolates used were archived strains from pure-culture MGIT 960 system tubes (Becton Dickinson, Franklin Lakes, NJ), with species identification and genotypic resistance to rifampin and isoniazid determined using the Genotype MTBDR*plus* assay (Hain Lifescience, Germany) according to the manufacturer's instructions. Phenotypic resistance for first- and second-line drugs was determined using the MGIT 960 system as previously described (17). Critical concentrations for ofloxacin and kanamycin (second-line drugs) were 2.0 μg/ml and 5.0 μg/ml, respectively. Resistance to first- and second-line drugs was determined using standard diagnostics algorithms as previously described (17, 27).

DNA preparation. *M. tuberculosis* isolates were handled in blinded fashion throughout. *M. tuberculosis* samples (0.5 ml) were pipetted into cryovial tubes containing 1.5 ml PrimeStore molecular transport medium (MTM; Longhorn Vaccines & Diagnostics, San Antonio, TX). PrimeStore MTM is a clinical transport solution that inactivates a broad range of microbes (5), including *M. tuberculosis* (8; A. W. Dreyer, N. A. Ismail, S. V. Omar, P. B. Fourie, K. Baba, and A. A. Hoosen, unpublished data), and preserves and stabilizes released RNA and DNA for safe ambient-temperature shipment (4, 5). Inactivated samples were transported from South Africa to San Antonio, TX, at ambient temperature (3 to 4 days) and stored at 5°C until used. Total DNA (50 μl) was purified from a 200-μl aliquot of PrimeStore MTM containing inactivated culture using a Qiagen EZ1 advanced robot and EZ1 DNA tissue kit (catalog no. 953034) according to the manufacturer's recommendations (Qiagen Inc., Germantown, MD).

Primer design. Novel PCR primers were designed for amplification of full-length coding regions for each *M. tuberculosis* gene of interest (Table 1). Primer pairs for *rpoB* (2 sets of primers), *katG*, *pncA*, *gyrA*, and *rrs* (16S) gene amplification were designed using the genome sequence of *M. tuberculosis* strain H37Rv (GenBank accession no. NC_000962) as a reference. Primer secondary structure, melting temperature, and potential primer-dimer formation were determined using LaserGene 9.1 (DNAStar, Madison, WI) and PrimerExpress 3.0 (Life Technologies, Foster City, CA). All oligonucleotides were synthesized using standard, desalted primers (Integrated DNA Technologies [IDT], San Diego, CA).

PCR amplification. Amplification reactions for all *M. tuberculosis* gene targets were designed and optimized to be used under one standardized set of thermocycling parameters. All PCR master mixes were prepared using Platinum *Taq* DNA polymerase, 10× buffer, and 50 mM $MgCl_2$ (part no. [PN] 10966-034; Life Technologies). Amplification was carried out in a 50-μl final volume reaction mixture containing 24.1 μl Ambion nuclease-free water (catalog no. AM 9932; Life Technologies), 5 μl 10× PCR buffer, 2 μl 50 mM $MgCl_2$ (2 mM final concentration), 0.4 μl PCR nucleotide mix ultrapure deoxynucleoside triphosphates (dNTPs) (200 μM final concentration for each dNTP, PN 77119; USB, Santa Clara, CA), 0.5 μl Platinum *Taq* DNA polymerase (2.5-U final concentration), and 2 μl primer blend (*rpoB*, *katG*, *pncA*, *gyrA*, or *rrs* gene; 0.4 μM final concentration for each primer). To each 34-μl master mix reaction mixture, 16 μl extracted DNA was added to bring the total volume to 50 μl. Reactions were carried out in MicroAmp optical 96-well reaction plates (PN N801-0560; Life Technologies) and capped using MicroAmp 8-cap strips (PN 4323032; Life Technologies). Amplification was performed using an ABI 2720 thermocycler (Life Technologies). Thermocycling parameters were 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 55°C for 15 s, and 72°C for 2 min, with final extension at 72°C for 5 min. Resulting amplicons were confirmed by addition of 5 μl PCR product with 1 μl GelPilot loading dye, 5× (PN 1037649; Qiagen), on 1% (wt/vol) molecular-biology-grade agarose (catalog no. BP1356; Fisher Scientific, Pittsburgh, PA) with ethidium bromide (0.1-μg/ml final concentration, catalog no. 161-0433; Bio-Rad, Hercules, CA). Electrophoretic separation of products was carried out for 60 min at 0.4 mV/cm^2 in 1× Tris-borate-EDTA (TBE) buffer (catalog no. 1B70153; IBI Scientific, Peosta, IA). Amplicons were visualized under UV transillumination, and size estimation was made using a TrackIt 1 kb Plus DNA Ladder (PN 10488-085; Life Technologies). After visualization, the remaining PCR mixtures for each clinical isolate gene amplification (~45 μl), corresponding to *rpoB*, *katG*, *pncA*, *gyrA*, and *rrs* (16S) targets, were transferred to a single microcentrifuge tube. Pooled genes corresponding to each clinical isolate were subjected to PCR purification and eluted in 50 μl low Tris-EDTA (TE) (catalog no. 602-1155-010; Life Technologies) using the MinElute reaction cleanup kit (catalog no. 28204; Qiagen) according to the manufacturer's instructions. The concentration and purity of DNA were determined spectrophotometrically using a NanoDrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE).

Ion Torrent library preparation. Bar-coded libraries were generated using the Ion Xpress Plus fragment library kit (catalog no. 4471269; Life Technologies) and the Ion Xpress DNA bar coding kit with adaptors 1 to 16 (catalog no. 4468654; Life Technologies) as described previously (10).

Amplicon shearing. Chemical shearing was performed using 1 to 3 μg DNA containing an approximate equimolar pool of *rpoB*, *katG*, *pncA*, *gyrA*, and *rrs* (16S) gene amplicons. DNA shearing was performed in a 50-μl total reaction volume by combining 5 μl Ion Shear Plus 10× reaction buffer, 10 μl enzyme, and 35 μl pooled DNA template (Ion Xpress Plus fragment library kit, catalog no. 4471269; Life Technologies). The reaction mixture was incubated at 37°C for 45 min, the reaction was terminated using 5 μl Ion Shear stop buffer, and the mixture was stored on ice until purification. Sheared DNA was purified using Agencourt Ampure XP-PCR purification beads (PN A63880; Beckman Coulter, Brea, CA) with a Dynal magnetic bead stand (catalog no. 123-21D; Life Technologies) according to the manufacturer's recommendations. Briefly, 99 μl Agencourt beads was mixed with 50 μl Ion Shear reaction mixture, incubated for 5 min at room temperature, placed on a magnetic stand, washed twice with 70% (vol/vol) ethanol, and eluted using 12 μl low-TE buffer (catalog no. 602-1155-010; Life Technologies).

Adaptor ligation. Adaptor ligation was performed in a 0.2-ml low-binding PCR tube (PN PCR-02-L-C; Axygen Inc., Union City, CA) by combining 12 μl sheared amplicon with 1.25 μl ligase buffer, 1.25 μl P1-IA adaptor mix (Ion DNA bar coding kit with adaptors 1 to 16; Life Technologies), and 0.2 μl DNA ligase (Ion Xpress Plus fragment library kit). The mixture was pipetted up and down 5 times and incubated at room temperature (22 to 25°C) for 30 min. Adaptor ligation reactions were purified and eluted in 10 μl low-TE buffer using Agencourt Ampure XP-PCR purification beads (Beckman Coulter) with the Dynal magnetic bead stand (Life Technologies) according to the manufacturer's recommendations.

Nick translation and bar code amplification. Amplicon pools from each patient sample were bar coded using the Ion DNA bar coding kit with adaptors 1 to 16 and Ion Xpress fragment library kit (Life Technologies). To maximize yields, reactions were scaled 2× by combining 40 μl Platinum PCR SuperMix High Fidelity, 4.4 μl Ion primer mix (BC X, where X = bar codes 1 to 16), and 10 μl ligated DNA. Amplification was performed using an ABI 2720 thermocycler (Life Technologies). Thermocycling parameters consisted of 72°C for 20 min and 95°C for 5 min, followed by 10 cycles at 95°C for 15 s, 58°C for 15 s, and 68°C for 1 min. Following amplification, bar-coded samples were purified and eluted in 50 μl low-TE buffer (Life Technologies) using the MinElute reaction cleanup kit (Qiagen) according to the manufacturer's instructions. DNA concentration and purity were determined spectrophotometrically using a NanoDrop ND 1000 (Thermo Fisher Scientific). Equimolar concentrations (~2 to 3 μg of each bar-coded sample) were combined into a single 1.5-ml nuclease-free microcentrifuge tube and subjected to size selection.

Size selection. The appropriate volume of GelPilot 5× loading dye (PN 1037649; Qiagen) was added to the pooled bar-coded *M. tuberculosis* library tube and loaded onto a 1% (wt/vol) agarose gel (catalog no. BP1356; Fisher Scientific, Pittsburgh, PA) containing ethidium bromide (0.1-μg/ml final concentration, catalog no. 161-0433; Bio-Rad, Hercules, CA). The bar-coded library was electrophoresed for 60 min at 0.4 mV/cm^2 in 1× TBE buffer (catalog no. 1B70153; IBI Scientific, Peosta, IA) and visualized under UV transillumination. Size estimations were determined using a TrackIt 1 kb Plus DNA ladder (Life Technologies). Gel excision was performed under UV transillumination using a sterile scalpel blade, excising out a target region between 75 and 200 bp. Excised agarose gel slices were placed into sterile 1.5-ml microcentrifuge tubes and subjected to DNA purification using the PureLink quick gel extraction kit (catalog no. K210012; Life Technologies) according to the manufacturer's instructions. Concentration and purity values for the bar-coded DNA library were determined spectrophotometrically using a NanoDrop ND 1000 (Thermo Fisher Scientific). The recommended library input for emulsion PCR is ~140 × 10^6 to 560 × 10^6 molecules per 18 μl. This range was achieved by a 1:1,000 dilution using library stock and nuclease-free water.

emPCR. Emulsion PCR (emPCR) was performed in a 1-ml reaction volume using the Ion template preparation kit (catalog no. 4469000; Life Technologies) by adding 582 μl nuclease-free water, 200 μl 5× PCR reagent mix, 100 μl 10× PCR enzyme mix, 100 μl Ion Sphere particles (ISPs), and 18 μl diluted library template. The preparation was mixed thoroughly, followed by brief centrifugation in a microcentrifuge. Emulsion was achieved using the Ultra-Turrax tube drive (Life Technologies). A total of 9 ml chilled emulsion oil (Ion template preparation kit; Life Technologies) was added to an Ion template preparation tube (catalog no. 4467226; Life Technologies). The emulsion tube was placed and locked onto the IKA Ultra-Turrax tube drive and initiated. While the tube was in motion, the entire 1-ml PCR master mix solution was dispensed into the cap port and mixed for 5 min. The mixed emulsion was transferred to a 96-well PCR plate and amplified using an ABI 2720 thermocycler (Life Technologies) using the following thermocycling parameters: 94°C for 6 min, followed by 40 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s and then 5 cycles at 94°C for 30 s and 68°C for 6 min.

ISP recovery and qubit measurement. Ion Sphere particles were recovered using reagents supplied in the Ion Xpress template kit (catalog no. 4469001; Life Technologies) according to the manufacturer's protocol (Ion Xpress template kit user guide, version 2.0, pages 18 and 19). Quantification of recovered particles was performed using a Qubit 2.0 fluorometer (Life Technologies) and an Ion Sphere quality control kit (catalog no. 4468656; Life Technologies) according to the manufacturer's recommendations (Ion Xpress template kit user guide, pages 25 and 26). The optimal amount of template-positive ISPs is between 4 and 50%. Relative fluorescent unit (RFU) values obtained outside this range were not pursued into subsequent ISP enrichment.

ISP enrichment. Enrichment of ISPs was achieved using reagents supplied in the Ion Xpress template kit, Ion sequencing kit, and DynaBeads MyOne streptavidin C1 beads (catalog no. 4469001, 4468997, and 650.01, respectively; Life Technologies) according to the manufacturer's protocols (Ion Xpress template kit user guide, version 2.0, pages 15 to 17).

Ion Torrent 314 chip preparation and PGM sequencing. Ion Torrent 314 chips (catalog no. 4462923; Life Technologies) were prepared and loaded according to the manufacturer's recommendation (Ion sequencing kit user guide, version 2.0). The Ion Torrent PGM was run according to Ion Torrent 314 chip specifications, including a 65-cycle sequencing protocol, use of 18 megaohms purified water, and standard compressed argon gas to drive fluidics through the PGM system. For each sequencing run, up to 16 clinical samples consisting of full-length genes (*rpoB*, *katG*, *pncA*, *gyrA*, and *rrs*) can be placed on a single 314 chip. Depending on the number of bar codes used, i.e., 1 to 16, a typical sequencing run produced an average base pair read length of 60 to 70 bp, a total of 250 to 300,000 high-quality (AQ20) reads, and gene coverage of 300 to 500 times.

Nucleotide sequence accession numbers. All sequences for the *rpoB*, *katG*, *pncA*, *gyrA*, and *rrs* genes and corresponding proteins were deposited into GenBank (accession numbers JX303203 to JX303332).

RESULTS

Phenotypic and genotypic results. Amino acid characterizations of 26 *M. tuberculosis* isolates by Ion Torrent sequencing of the *rpoB*, *katG*, *pncA*, *gyrA*, and *rrs* (16S) genes are summarized in Tables 2 to 6, respectively, and compared to results obtained by Bactec MGIT 960 (phenotypic) and/or Hain GenoType MTBDR*plus* (genotypic) LPA. Of the 26 *M. tuberculosis* clinical isolates, 14 (54%) were MDR, 7 (27%) were XDR, and 5 (19%) were susceptible to drugs by Bactec MGIT 960 phenotypic analysis. The Ion Torrent PGM sequencing method showed 100% (26/26) concordance to both phenotypic resistance obtained by MGIT 960 culture (Tables 2 to 6) and genotypic *rpoB* and *katG* data obtained by Hain LPA (Tables 2 and 3).

TABLE 2 Summary of 10 amino acid mutations in the first 900 amino acid residues[a] of the *rpoB* gene products of 26 (14 MDR, 7 XDR, and 5 fully susceptible) *M. tuberculosis* isolates from South Africa deduced by Ion Torrent sequencing, Hain LPA genotyping, and culture

No. of isolates	Amino acid substitution(s)[b] yielded by the *rpoB* gene (3,619 bp)	Rifampin result by:		
		Ion Torrent[c]	Hain LPA	Bactec MGIT 960
9	**S531L**	Resistant	Resistant	Resistant
1	**S531L**, V194I	Resistant	Resistant	Resistant
1	**S531L,** Y645H	Resistant	Resistant	Resistant
1	**H526D**	Resistant	Resistant	Resistant
1	**H526Y**, S509R	Resistant	Resistant	Resistant
1	**H526R**	Resistant	Resistant[d]	Resistant
5	Wild type[b]	Sensitive	Sensitive	Sensitive
6	**D516G**, L533P	Resistant	Resistant[d]	Resistant
1	**D516V**	Resistant	Resistant	Resistant

[a] There were 5 *rpoB* mutations yielding amino acid substitutions (R908C, Q1042H, P1043A, I1187T, and V1249F) noted in at least 1 strain at the 3′ end (residues 900 to 1253).
[b] Compared to the H37Rv reference strain.
[c] Rifampin resistance is known to occur in *rpoB* at positions corresponding to amino acids 531(S→T), 526 (H→Y/D), and 516 (D→V).
[d] Using Hain LPA, strains with an uncommon glycine substitution at position 516 and an arginine at position 526 were indirectly determined as resistant by absence of a wild-type band.

***rpoB* gene mutations.** A total of 10 *rpoB* amino acid substitutions were identified in the 26 clinical isolates compared to the H37Rv wild-type strain. The common S531L mutation was the most prevalent, but mutations at positions 516 and 526, also known to confer resistance to rifampin, were observed (Table 2). Additionally, mutations were observed within the *rpoB* open reading frame but outside the 81-bp rifampin resistance-determining region (RRDR) (Table 2). The V194I mutation observed outside the RRDR in one strain is a unique substitution that is likely not associated with rifampin resistance. Five amino acid substitutions were noted in at least one strain beyond residue 900 of the RpoB protein. There were seven strains with an RpoB mutation (6 at position 516 and 1 at position 526) where a wild-type band was absent without a corresponding mutation band according to LPA. In six of these seven isolates, Ion Torrent sequencing revealed an uncommon amino acid substitution (i.e., glycine) within a known mutation site at position 516, where a valine substitution (D516V) is typically known to occur (Table 2). Similarly, in one isolate Ion Torrent sequencing revealed an arginine within a known mutation site at position 526 where tyrosine or aspartic acid substitutions (H526Y/D) typically occur.

TABLE 3 Summary of 4 amino acid mutations in the *katG* gene products of 26 (14 MDR, 7 XDR, and 5 fully susceptible) *M. tuberculosis* isolates from South Africa deduced by Ion Torrent sequencing, Hain LPA genotyping, and culture

No. of isolates	Amino acid substitution(s)[a] yielded by the *katG* gene (2,447 bp)	Isoniazid result by:		
		Ion Torrent[b]	Hain LPA	Bactec MGIT 960
11	**S315T**	Resistant	Resistant	Resistant
5	**S315T**, R463L	Resistant	Resistant	Resistant
1	W191R, R463L	Sensitive	Sensitive	Sensitive
7	Wild type[a]	Sensitive	Sensitive	Sensitive
1	R463L	Sensitive	Sensitive	Sensitive
1	N138H	Sensitive	Sensitive	Sensitive

[a] Compared to the H37Rv reference strain.
[b] Isoniazid resistance is known to occur in the *katG* product at position 315 (S→T) (indicated in bold).

TABLE 4 Summary of 6 amino acid mutations in the *pncA* gene products of 26 (14 MDR, 7 XDR, and 5 fully susceptible) *M. tuberculosis* isolates from South Africa deduced by Ion Torrent sequencing and culture

No. of isolates	Amino acid substitution(s)[a] yielded by the *pncA* gene (3,619 bp)	Pyrazinamide result by:	
		Ion Torrent[b]	Bactec MGIT 960
3	**C14R**	Resistant	Resistant
1	**A102V**	Resistant	Resistant
1	**Q122Stop**	Resistant	Resistant
16	Wild type[a]	Sensitive	Sensitive
1	**V139G**	Resistant	Resistant
1	**R154G**	Resistant	Resistant
2	**L172P**	Resistant	Resistant
1	Silent (C195T)[c]	Sensitive	Sensitive

[a] Compared to the H37Rv reference strain.
[b] Pyrazinamide resistance is known to occur in several mutations described by Mphahlele et al. (13) (indicated in bold).
[c] One strain contained a silent (synonymous) nucleotide mutation corresponding to position 195 (C→T).

***katG* gene mutations.** Four amino acid substitutions were observed in the *katG* gene product, with S315T, which is known to confer isoniazid resistance, present in all resistant strains (Table 3). Clinical strains harboring R463L, W191R, and N138H mutations were detected by DST (Table 3) and have been previously characterized. A substitution at position 463 (R463L) in the *katG* product has been previously shown to have no effect on antibiotic resistance and can be used to categorize *M. tuberculosis* isolates into genetic groups 1 (Arg463) and 2 or 3 (Leu463). Of the 26

TABLE 5 Summary of 10 amino acid mutations in the *gyrA* gene products of 26 (14 MDR, 7 XD, and 5 fully susceptible) *M. tuberculosis* isolates from South Africa deduced by Ion Torrent sequencing and culture

No. of isolates	Amino acid substitution(s)[a] yielded by the *gyrA* gene (2,664 bp)	Fluoroquinolone result by:	
		Ion Torrent[b]	Bactec MGIT 960
3	E21Q, S95T, G247S, G668D	Sensitive	Sensitive
2	E21Q, **D94G**, S95T, G668D	Resistant	Resistant
1	E21Q, **G88C**, S95T, G688D	Resistant	Resistant
10	E21Q, S95T, G668D	Sensitive	Sensitive
1	Wild type[a]	Sensitive	Sensitive
1	E21Q, S95T, G668D, Q613Q/E[c]	Sensitive	Sensitive
1	E21Q, S95T, G668D, L549S/L[c]	Sensitive	Sensitive
1	E21Q, **D94Y**, S95T, G668D	Resistant	Resistant
6	E21Q, **A90V**, S95T, G247S, G668D	Resistant	Resistant

[a] Compared to the H37Rv reference strain.
[b] Fluoroquinolone resistance is known to occur in the *gyrA* gene product at positions 88 (G→C), 90 (A→V), 91 (S→P), and 94 (D→H) (indicated in bold).
[c] There is a heterozygous nucleotide mutation in a proportion of Ion Torrent reads; the mutation confers a mixed amino acid substitution.

TABLE 6 Summary of 4 amino acid mutations in the *rrs* (16S) gene products of 26 (14 MDR, 7 XDR, and 5 fully susceptible) *M. tuberculosis* isolates from South Africa deduced by Ion Torrent sequencing and culture

No. of isolates	Amino acid substitution(s)[a] in the *rrs* (16S) gene (1,680 bp)	Kanamycin result by:	
		Ion Torrent[b]	Bactec MGIT 960
1	G878A	Sensitive	Sensitive
12	Wild type[a]	Sensitive	Sensitive
1	A514C, **A1401G**	Resistant	Resistant
6	**A1401G**	Resistant	Resistant
3	A514C	Sensitive	Sensitive
1	C492T	Sensitive	Sensitive
1	C492T, A514C	Sensitive	Sensitive
1	A514C	Sensitive	Sensitive

[a] Compared to the H37Rv reference strain.
[b] Aminoglycoside resistance is known to occur at positions 1401 (A→G), 1402 (C→T), and 1484 (G→T) (indicated in bold).

clinical isolates assessed, 7 (27%) were members of genetic group 1 as evident by this R463L substitution.

***pncA* gene mutations.** Seven nucleotide mutations were noted in at least one strain among 561 bp comprising the full-length coding region for the *pncA* gene (Table 4). Nine of 26 strains (34.6%) contained an amino acid mutation conferring pyrazinamide (PZA) resistance (Table 4). In one strain, a silent (synonymous) nucleotide mutation was characterized at a position corresponding to amino acid 195 (C195T). Five strains contained previously characterized amino acid substitutions (C14R, A102V, V139G, R154G, and L172P) known to confer resistance to pyrazinamide. A novel mutation, not previously reported elsewhere, including a termination codon was found in one isolate at residue 122 (Q122Stop) in the PncA protein (Table 4).

***gyrA* gene mutations.** Nine unique mutations were observed in the 2,517-bp full-length *gyrA* gene encoding subunit A of the DNA gyrase enzyme. Resistance to fluoroquinolones (FQs) was noted only in strains harboring mutations in the quinolone resistance-determining region (QRDR) defined by substitutions in *gyrA* at positions corresponding to amino acids 88, 90, and 94. A number of additional mutations were also observed in regions outside the QRDR, including two "mixed strain" mutations at positions 549 and 613 in the GyrA protein (Table 5). Mutation at position 95 (S95T) is known to have no effect on FQ resistance but can be used to categorize strains in genetic group 2 or 3. Of the 19 total clinical isolates belonging to genetic groups 2 and 3, 18 (96%) were group 2 and 1 (4%) was group 3 according to assessment of GyrA position 95 (T indicates genetic group 2 and S indicates genetic group 3).

***rrs* (16S) gene mutations.** Four nucleotide mutations were noted among the 1,540 bp comprising the full-length 16S *rrs* gene. Seven of the 26 (27%) clinical isolates were shown to be resistant to aminoglycosides by DST, and all strains harbored an A1401G mutation known to confer resistance (Table 6). Two other amino acid mutations (C492T and A514C) were observed but have been previously shown to not inhibit aminoglycoside efficacy. A previously uncharacterized G878A nucleotide mutation was observed, but the isolate was shown to be susceptible according to DST (Table 6).

DISCUSSION

This study describes the first standardized protocol utilizing Ion Torrent sequencing of full-length genes to characterize MDR and XDR *M. tuberculosis* strains. The protocol described in this report enables sequencing of entire coding regions implemented in *M. tuberculosis* resistance, allowing characterization of known mutations and discovery of new polymorphisms.

Rapid analysis of genes associated with drug-resistant strains is a major challenge for successful treatment of tuberculosis. In addition, real-time geographical surveillance of emerging *M. tuberculosis* drug resistance would facilitate more appropriate antibiotic treatment strategies. Currently, available molecular methods such as the GenoType MTBDR*plus* LPA offer limited detection capabilities, particularly when novel or uncommon amino acid substitutions are within known drug resistance regions or when undiscovered amino acid mutations impact drug resistance. We have established a simplified Ion Torrent sequencing protocol for rapid characterization of five full-length genes (cumulatively sequencing 11.4 kb per isolate) for determining drug resistance in MDR and XDR strains.

The *M. tuberculosis rpoB* gene encodes the 1,178-amino-acid beta subunit for a DNA-dependent RNA polymerase enzyme. Mutations within an 81-bp "core region" of the *rpoB* gene are responsible for approximately 95% of rifampin resistance in *M. tuberculosis* strains (24). Three of these mutations, yielding substitutions at positions 516 (D→V), 526 (H→Y/D), and 531 (S→L), constitute the majority of mutations within this region. Of the 21 rifampin-resistant strains characterized in this study, 11 (52.4%) carried the S531L mutation, 7 (33.3%) contained an amino acid substitution at position 516, and 3 (14.3%) contained a mutation at position 526 of the *rpoB* gene product (Table 2). The most prevalent RpoB substitution observed at position 516 is a valine (D516V) (26). Interestingly, Ion Torrent sequencing revealed that 6 of 7 strains contained a rarer glycine residue (D516G) at this position (Table 2). Using LPA, these 6 strains were determined to be resistant, since both mutant and wild-type bands were absent (Table 2). The D516G mutation occurs less frequently than D516V, is not clustered or epidemiologically significant, and has been reported to occur in isolates characterized from China, Africa, and North America (7, 12, 23). Similarly, an uncommon amino acid substitution was identified at position 526 in the *rpoB* gene product. The most prevalent amino acid substitution reported at position 526 in the *rpoB* gene product is a change from histidine to tyrosine or aspartic acid (H526Y/D) (24, 26). However, Ion Torrent sequencing revealed that 1 of 3 isolates contained an uncommon arginine residue (H526R) that by Hain LPA was shown to be absent for both wild-type and mutant bands (Table 2). While the absence of wild-type and mutant bands in a sample is interpreted as resistant according to LPA testing (6), there remains ambiguity, since the type of amino acid change is not directly characterized. The absence of wild-type and mutant bands as an indication of drug resistance using the LPA test could possibly give a false-positive result if different amino acid residues in known amino acid positions affect resistance or levels of resistance to a particular drug. This underscores the utility of Ion Torrent sequencing for directly detecting specific amino acid substitutions in positions known to confer resistance, as well as discovery of novel amino acids outside known sites that may impact resistance.

The *katG* gene encodes catalase peroxidase, an enzyme that converts isoniazid (INH) into the active form. The majority of isoniazid resistance is associated with the *katG* codon corresponding to amino acid 315 (S315T) (9), although mutations in the promoter region of *inhA* and *nod* also contribute to resistance (9, 15). Of the 26 strains assessed, 16 contained the characteristic serine-to-threonine amino acid substitution at position 315 (S315T) conferring isoniazid resistance (Table 3). These sequencing results exhibited 100% concordance with comparisons made using the Hain LPA and culture DST.

Pyrazinamide (PZA) is a synthetic derivative of nicotinamide that has been used as a first-line drug to fight tuberculosis since 1952. Standard DST for PZA is complicated due to an acidic pH requirement *in vitro*, which inhibits *M. tuberculosis* growth and complicates accurate phenotypic assessment. PZA resistance is attributed to mutations in the *pncA* gene, which encodes a pyrazinamidase. However, these resistance-conferring mutations are numerous and consist of amino acid substitutions, frameshifts, and stop codon mutations (13). Seven mutations were characterized from the 26 South African isolates assessed, including one silent mutation, five amino acid substitutions, and one chain termination mutation. The Q122Stop termination mutation (Table 4) observed in one isolate is novel, having never been reported elsewhere. The difficulty in PZA phenotypic assessment and the variability of mutations along the *pncA* gene highlight the use of Ion Torrent gene sequencing to assess mutations in this hypervariable *M. tuberculosis* gene.

The primary target of fluoroquinolones (FQs) in *M. tuberculosis* is DNA gyrase, a type II topoisomerase composed of two A and B subunits encoded by the *gyrA* and *gyrB* genes, respectively (2). Amino acid substitutions located within a short region of the *gyrA* gene known as the quinolone resistance-determining region (QRDR) account for the majority of known FQ-resistant *M. tuberculosis* strains (22). Substitution mutations in the QRDR at positions 88, 90, and 94 were observed in 10 of 26 sequences from this study (Table 5). Three of the 10 strains contained substitutions at position 94 in the *gyrA* gene product; two were noted as D94G substitutions, and one was a D94Y substitution. A previous study characterized both D94G and D94Y substitutions and demonstrated that these amino acid substitutions at position 94 give rise to similar levels of FQ resistance (3). Among the strains assessed in this study, the *gyrA* gene was the most variable, yielding nine amino acid substitutions in the 26 clinical isolates assessed. Furthermore, two of these *gyrA* codons (corresponding to amino acids 549 and 613) exhibited heterogeneous residues (Table 5), an advantage of performing Ion Torrent sequencing over Hain LPA and DST.

Emerging cases of XDR tuberculosis, defined as MDR cases having acquired additional resistance to FQs, e.g., ofloxacin, and at least one of the second-line injectable drugs, e.g., amikacin (AMK), kanamycin (KAN), or capreomycin (CAP), have become a public health threat in developing countries worldwide (11). The majority of resistance to second-line drugs is associated with mutations in codons corresponding to amino acids 1401 (A1401G), 1402 (C1402T), and 1484 (G1484T) in the 16S rRNA *rrs* gene (11, 21). Analysis of African *M. tuberculosis* strains revealed that 7 of the 26 (27%) were defined as XDR as evident by mutation at position 1401 (A1401G) (Table 5). Three additional nucleotide mutations affecting positions 492, 514, and 878 were also discovered (Table 6) in strains from this analysis. G878A is a novel mutation, but the strain carrying it was shown to be susceptible to AMK, KAN, and CAP according to DST (data not shown). The overall cumulative impact of these newly discovered mutations on drug resistance remains to be established.

Previous studies have shown that mutations in the *katG* codon corresponding to amino acid 463 and the *gyrA* codon corresponding to amino acid 95 are genetic markers for categorizing strains into epidemiological genetic groups 1, 2, and 3 and that these codons have no effect on antibiotic resistance (16, 18). Group 1 strains are genetic ancestors of group 2 and group 3 strains that link the predominately nonhuman *Mycobacterium* species (*M. microti* and *M. bovis* strains) with human *M. africanum* and *M. tuberculosis* lineages (21). As evident by substitution mutations in the *katG* codon corresponding to amino acid 463 and in the *gyrA* codon corresponding to amino acid 95, a total of 7 of 26 (27%), 18 of 26 (69%), and 1 of 26 (4%) African isolates characterized in this study were members of genetic groups 1, 2, and 3, respectively. Tracking group 1 organisms is important in terms of *M. tuberculosis* detection, since several isolates belonging to genetic group 1 lack insertion sequence 6110 (IS*6110*) (18), a common genetic target for several PCR-based *M. tuberculosis* detection assays.

The purpose of this article was to describe the development of an Ion Torrent protocol for evaluating genotypic drug resistance using clinical isolates. In this study, we characterized a very limited set of MDR, XDR, and drug-susceptible clinical isolates from two provinces in South Africa. We intend to apply our sequencing methodology to a much larger prospective study using samples collected from Kwa-Zulu Natal Province in South Africa. Evaluation of a larger sample size will further validate the developed methodology and make possible detection of uncommon or novel mutations in the full coding regions of these five genes.

One inherent concern with performing full-gene sequencing, especially from geographically diversified samples, is the discovery of novel mutations that may have no impact on drug susceptibility. Conversely, detected mutations outside known resistance sites may cause complete resistance or an additive resistance when combined with other mutations. Thus, genotypic identification of mutations through full-gene sequencing is the first step in understanding the complete resistance picture. However, genotypic discovery might be complemented and validated by subsequent phenotypic MIC studies.

There is potential for the Ion Torrent instrument and described methodology to be integrated into selected regional and reference settings throughout Africa, India, and China. In contrast to most Sanger sequencers, the Ion Torrent does not utilize (expensive and maintenance-requiring) lasers that typically require modified fluorescence-based, light-sensitive chemistries, and it has a much smaller overall footprint. Furthermore, our described methodology does not require expensive ancillary equipment such as the Agilent 2100 BioAnalyzer, the Ion OneTouch system, ultracentrifuges, or the Pippin Prep Workstation as current Ion Torrent protocols recommend. This is significant, since these instruments and needed accessories and consumables can be expensive, require large laboratory footprints, and often require routine maintenance. Additionally, since our protocol employs an initial PCR amplification for full-length genes (not full genomes), we do not require the DiaGenode Bioruptor Sonication System for DNA shearing. When clinical isolates derived from traditional detection methods require further genotypic drug susceptibility characterization, they can safely be inactivated in PrimeStore MTM for

extraction and PCR amplification in a standard molecular biology laboratory setting prior to Ion Torrent sequencing. Using our described methodology for characterization of 5 full-length genes, between 1 and 16 patient libraries can be sequenced and analyzed within 2 days for $75 to $80 per patient; these costs will likely be reduced as the technology matures.

In contrast to the GenoType MTBDR*plus* or MTBDRsl LPA, the Ion Torrent PGM protocol reported here provides full-length characterization of genes, making possible discovery of new amino acid substitutions that could potentially be missed by LPA, since LPA is limited to only known mutations. Using the protocol reported here, several uncommon amino acid changes in clinical field isolates have been found. Furthermore, the extensive depth of sequence coverage from the Ion Torrent allows for discovery of heterogeneous or mixed-strain genetic populations within an isolate.

The scalability of Ion Torrent sequencing permits expansion to include megabases of additional genes on a single chip. As a proof of principle with five full-length *M. tuberculosis* genes, our methodology could easily be expanded to include all 16+ genes that currently constitute *M. tuberculosis* drug resistance. Full-length gene analysis using the Ion Torrent PGM may identify novel mutations that, when correlated to phenotypic MIC testing, could identify new *M. tuberculosis* resistant residues as well as the cumulative inhibitory effect of multiple mutations.

ACKNOWLEDGMENTS

This work would not have been possible without the efforts of Chris Helm and Susan Graham, who facilitated international planning meetings, travel, and teleconferences between U.S. and South African collaborators. We thank Richard Tamfu for his critical review of the manuscript. We are also appreciative of Matthew (Matt) Keyser from DNAStar Inc. (Madison, WI) for his bioinformatics assistance.

REFERENCES

1. **Adjers-Koskela K, Katila ML.** 2003. Susceptibility testing with the manual Mycobacteria Growth Indicator Tube (MGIT) and the MGIT 960 system provides rapid and reliable verification of multidrug-resistant tuberculosis. J. Clin. Microbiol. **41**:1235–1239.
2. **Aubry A, Pan XS, Fisher LM, Jarlier V, Cambau E.** 2004. *Mycobacterium tuberculosis* DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity. Antimicrob. Agents Chemother. **48**: 1281–1288.
3. **Cui A, Wan J, Lu J, Huang X, Hu Z.** 2011. Association of mutation patterns in *gyrA/B* genes and ofloxacin resistance levels in *Mycobacterium tuberculosis* isolates from East China in 2009. BMC Infect. Dis. **11**:78.
4. **Daum LT, et al.** 2012. Genetic analysis of Mycobacterium tuberculosis drug-resistance genes from samples shipped in PrimeStore MTM and sequenced using the next-generation Ion Torrent, abstr 29570. 30th Annu. Meet. Eur. Soc. Paediatr. Infect. Dis. (ESPIDS), Thessaloniki, Greece, 8 to 12 May 2012. http://espid.ekonnect.co/ESPID_385/poster_29570/program.aspx.
5. **Daum LT, et al.** 2010. A clinical specimen collection and transport medium for molecular diagnostic and genomic applications. Epidemiol. Infect. **16**:1–10.
6. **Hillemann D, Weizenegger M, Kubica T, Richer E, Niemann S.** 2005. Use of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. J. Clin. Microbiol. **43**:3699–3703.
7. **Ioerger TR, et al.** 2009. Genome analysis of multi- and extensively-drug-resistant tuberculosis from KwaZulu-Natal, South Africa. PLoS One **4**:e7778. doi:10.1371/journal.pone.0007778.
8. **Ismail NA, et al.** 2011. Evaluation of PrimeStore molecular transport medium for use in the diagnosis of TB, abstr 2473. Eur. Soc. Clin. Microbiol. Infect. Dis. (ESSMID), 7 to 10 May 2011, Milan, Italy.
9. **Kim SY, et al.** 2004. Molecular analysis of isoniazid resistance in *Mycobacterium tuberculosis* isolates recovered from South Korea. Diagn. Microbiol. Infect. Dis. **47**:497–502.
10. **Life Technologies Incorporated.** 7 October 2011. Ion Xpress Plus gDNA and amplicon library preparation. User's guide. Version number 4471989, revision B. Life Technologies Incorporated, Foster City, CA.
11. **Marwar A, et al.** 2011. Extensively drug resistant tuberculosis (XDR-TB): a potential threat. J. Basic Clin. Pharm. **2**(1):27–32.
12. **McCammon MT, et al.** 2005. Detection of *rpoB* mutations associated with rifampin resistance in *Mycobacterium tuberculosis* using denaturing gradient gel electrophoresis. Antimicrob. Agents Chemother. **49**:2200–2209.
13. **Mphahlele M, et al.** 2008. Pyrazinamide resistance among South African multidrug-resistant *Mycobacterium tuberculosis* isolates. J. Clin. Microbiol. **46**:3459–3464.
14. **Pfyffer GE, Palicova F, Rüsch-Gerdes S.** 2002. Testing of susceptibility of *Mycobacterium tuberculosis* to pyrazinamide with the nonradiometric BACTEC MGIT 960 system. J. Clin. Microbiol. **40**:1670–1674.
15. **Ramaswamy SV, et al.** 2003. Single nucleotide polymorphisms in genes associated with isoniazid resistance in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **47**:1241–1250.
16. **Rouse DA, DeVito JA, Li Z, Byer H, Morris SL.** 1996. Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosis*: effects on catalase-peroxidase activities and isoniazid resistance. Mol. Microbiol. **22**: 583–592.
17. **Siddiqi SH.** 2006. MGIT procedure manual. Bactec MGIT 960 TB system. Becton Dickinson, Sparks, MD.
18. **Sreevatsan S, et al.** 1997. Restricted structural polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc. Natl. Acad. Sci. U. S. A. **94**:9869–9874.
19. **Statistics South Africa.** 2008. Mortality and causes of death in South Africa, 2008: findings from death notification. Statistics South Africa, Pretoria, South Africa. http://www.statssa.gov.za/publications/P03093/P030932008.pdf.
20. **Streicher EM, et al.** 2012. Emergence and treatment of multidrug resistant (MDR) and extensively drug-resistant (XDR) tuberculosis in South Africa. Infect. Genet. Evol. **12**:686–694.
21. **Suzuki T, et al.** 1998. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. J. Clin. Microbiol. **36**:1220–1225.
22. **Takiff HE, et al.** 1994. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. Antimicrob. Agents Chemother. **38**:773–780.
23. **Tan Y, et al.** 2012. The beginning of the *rpoB* gene in addition to the rifampin resistance determination region might be needed for identifying rifampin/rifabutin cross-resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates from Southern China. J. Clin. Microbiol. **50**:81–85.
24. **Telenti AP, et al.** 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet **341**:647–650.
25. **UNAIDS.** 2008. 2008 report on the global AIDS epidemic. UNAIDS, Geneva, Switzerland. http://data.unaids.org/pub/GlobalReport/2008/jc1511_gr08_executivesummary_en.pdf.
26. **Williams DL, et al.** 1994. Characterization of rifampin resistance in pathogenic mycobacteria. Antimicrob. Agents Chemother. **38**:2380–2386.
27. **World Health Organization.** 2001. Guidelines for drug susceptibility testing for second-line anti-tuberculosis drugs for DOTS-Plus. WHO/CDS/TB/2001.288. World Health Organization, Geneva, Switzerland.
28. **World Health Organization.** 2011. Towards universal access to diagnosis and a treatment of multidrug-resistant and extensively drug-resistant tuberculosis by 2015: WHO progress report 2011. WHO/HTM/TB/2011.3. World Health Organization, Geneva, Switzerland.
29. **World Health Organization.** 2011. Global tuberculosis control. WHO/HTM/TB/2011.16. World Health Organization, Geneva, Switzerland.
30. **Zignol M, et al.** 2006. Global incidence of multidrug-resistant tuberculosis. J. Infect. Dis. **194**:479–485.