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BRIEF REPORT

Cryptic Resistance Mutations Associated With Misdiagnoses of Multidrug-Resistant Tuberculosis

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Understanding why some multidrug-resistant tuberculosis cases are not detected by rapid phenotypic and genotypic routine clinical tests is essential to improve diagnostic assays and advance toward personalized tuberculosis treatment. Here, we combine whole-genome sequencing with single-colony phenotyping to identify a multidrug-resistant strain that had infected a patient for 9 years. Our investigation revealed the failure of rapid testing and genome-based prediction tools to identify the multidrug-resistant strain. The false-negative findings were caused by uncommon rifampicin and isoniazid resistance mutations. Although whole-genome sequencing data helped to personalize treatment, the patient developed extensively drug-resistant tuberculosis, highlighting the importance of coupling new diagnostic methods with appropriate treatment regimens.

Keywords. Tuberculosis; drug resistance; whole-genome sequencing; individualized treatment; cryptic mutations.

Personalized treatment in tuberculosis can be achieved in the next few years if we are able to implement rapid, cost-effective, and comprehensive drug susceptibility tests (DSTs). However, the prospects for this personalization deeply depend on our ability to identify drug resistance–associated mutations and to interpret their clinical role during management of the cases [1]. Current methods to identify and manage drug resistance are based on rapid liquid culture systems and/or molecular amplification tests [2]. Both approaches have limitations. For rifampicin (RIF), some mutations, termed "disputed" mutations [3], are systematically missed by rapid automatic liquid culture methods, such as the Bactec–Mycobacteria Growth Indicator Tube (Bactec-MGIT) system, which is used in this study. These noncanonical RIF resistance mutations are involved in low-level resistance and associated with relapse [2, 4]. Likewise, the number of mutations screened by nucleic acid amplifications tests is limited [3]. Until now, no case has been reported in which both the Bactec-MGIT system and genotypic assays failed to identify multidrug-resistant strains. Here, we report a multidrug-resistant strain with cryptic mutations not detected by rapid routine clinical methods or whole-genome prediction tools. Prospective whole-genome sequencing (WGS) helped to track additional resistance mutations, but failure to provide an appropriate treatment regimen led to extensively drug-resistant tuberculosis. Despite limited therapeutic options, the patient was declared cured in 2018.

METHODS

Clinical and microbiological data, together with a more-detailed description of the methods, are included in the Supplementary Data.

Clinical Case, Isolate Collection, and Routine DST Procedures

The study case was a Spanish-born patient with no common tuberculosis risk factors whose first episode of tuberculosis occurred in 2009. Findings of sputum and culture analyses became negative within 2 months after treatment initiation, and the patient was considered cured 4 months later, based on World Health Organization guidelines. However, relapse occurred in 2013 despite no risk factor for relapse during the initial episode [5]. Two years later, the patient was not responding to therapy despite compliance with treatment, close monitoring, and infection with a drug-susceptible strain, based on results of the hospital's routine rapid phenotypic DST and genotypic testing. We analyzed 16 serial clinical isolates recovered from the patient during 2009–2018 by the clinical microbiology unit of the Hospital Universitario General de Valencia (Valencia, Spain).

Routine phenotypic DST for the first-line antituberculosis drugs (and linezolid in 1 isolate) was performed on all clinical samples collected during the study period, using the Bactec-MGIT 960 system (Becton Dickinson, Franklin Lakes, NJ). For second-line drugs, the Sensititre MycoTB MIC plate (Trek Diagnostics System, Cleveland, OH) was used. Ranges of the critical concentrations for all drugs are specified in the Supplementary Data.

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WGS Analysis

Extracted DNA from diagnostic cultures was sequenced on the MiSeq platform, using standard procedures. We used Kraken [6] to identify reads belonging to *Mycobacterium tuberculosis* complex. For mapping and variant calling, we used a previously described pipeline [7]. For details, see the Supplementary Data.

Identification of Candidate Drug Resistance Variants

We identified candidate drug resistance variants by mapping them to known drug resistance–associated genes and confirming that they had not previously been described as phylogenetic markers (Supplementary Table 1). In addition, we screened any new variant that arose during the course of treatment in any part of the genome and reached a minimal frequency of 15% in ≥1 sample, to evaluate their potential role in drug resistance. Our in-house results were compared to data from 3 publically genomic resistance databases (accessed April 2018) [8–10]. The global frequencies of these candidate mutations were evaluated against an in-house database of 4762 genomes collected worldwide.

Isolation of Single Clones, Amplicon Sequencing, and Minimum Inhibitory Concentration (MIC) Validation for Resistance Mutations

After we identified mutations in genes or genomic regions associated with drug resistance to INH and RIF, we tested whether those variants conferred resistance, by characterizing a series of single-colony isolates. Twenty-two single clones with different genotypes, obtained from complex diagnostic cultures at different time points, were selected and isolated. Each clone was tested twice for susceptibility to INH, using the resazurin microtiter assay with 2-fold dilutions for 9 different concentrations (range, 0.06–32 μg/mL). We also confirmed that the I491F mutation conferred resistance to RIF, using the proportions method with 2-fold dilutions for 10 different concentrations (range, 0.06–64 μg/mL). Before DST, we performed ultra-deep amplicon sequencing of the regions of interest (*rpoB*, *katG*, and the *ahpC* promoter) to confirm the genotype of each clone, as well as to discount the presence of any unnoticed mutation with a frequency of ≥0.1%, the lower limit of detection (Supplementary Methods and Supplementary Table 6).

RESULTS

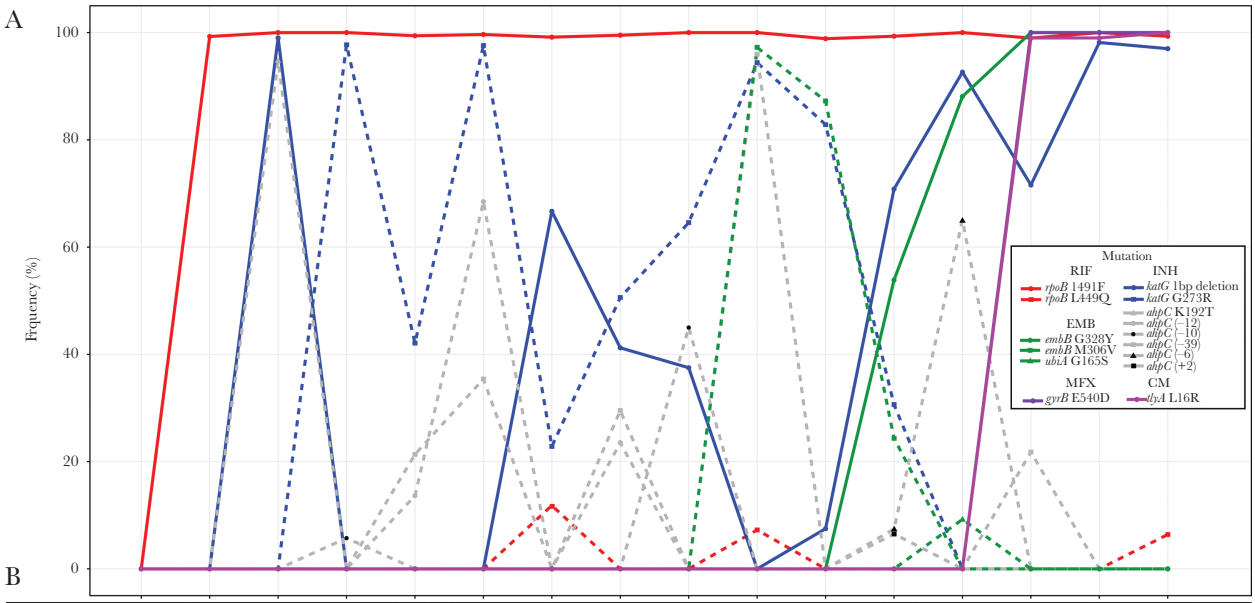
Cryptic Variants behind an Unnoticed Multidrug-Resistant Tuberculosis Case

A total of 16 isolates from the patient were available and sequenced during the study period (2009–2017). It is important to note that the first genome sequence was analyzed in 2015, after the patient had received standard first-line treatment for 2 years without a response (Supplementary Figure 1). Reconstruction of a phylogeny from WGS data strongly supported a scenario in which the relapse infection (which began in 2013) was caused by the strain from the first episode (which began in 2009; Supplementary Figure 2).

Inspection of candidate variants only revealed likely mutations in known drug resistance genes (a complete list is shown in Supplementary Table 2). We found the *rpoB* mutation I491F, which is a noncanonical but known RIF resistance–associated variant. I491F is systematically missed by the Bactec-MGIT system because of an unfortunate combination of slow mycobacterial growth and the system's switch to an automated readout after 20 days.

WGS analysis of previous isolates revealed that the I491F variant appeared to be fixed in the isolate initially cultured during the relapse episode (in September 2013) but not in the isolate from the first episode (in 2009). Thus, during the relapse episode, the mycobacteria were already resistant to RIF at the time the first positive culture result was obtained (Figure 1). Knowing this, we looked for INH resistance variants in the first isolate from the relapse episode, but we did not detect any putative mutation. However, in later isolates we identified 2 new noncanonical, mutually exclusive candidate INH resistance mutations, in *katG* (G249del and G273R). The G249del variant appeared as early as January 2014 but with highly variable frequency across samples, although it dominated the last mycobacteria-positive cultures (from June 2016 onward). In contrast, the G273R mutation appeared for the first time in March 2014, disappearing by December 2015. As expected for noncanonical *katG* mutations, screening of the *ahpC* gene and promoter region identified multiple *ahpC* promoter mutations whose presence fluctuated through time (Figure 1).

Switching treatment from a first-line regimen to a multidrug-resistant tuberculosis regimen is a major clinical decision. Thus, resistance variants detected by our genomic analyses needed validation. We selected 22 single clones from secondary cultures of specimens obtained at different time points during treatment and performed DST with alternative methods (Figure 2). Furthermore, we performed ultra-deep amplicon sequencing in specific RIF and INH resistance regions (Supplementary Methods). First, we confirmed that all 19 clones harboring the *rpoB* I491F mutation were RIF resistant (MIC, > 1 μg/mL) as compared to the 3 clones from 2009 with no mutation (MIC, < 1 μg/mL), which had an MIC higher than that for H37Rv but similar to that for other RIF-susceptible strains described elsewhere [11]. In the case of INH resistance, clones from 2009 and 2013 had a low MIC for INH (< 0.25 μg/mL), consistent with the fact that no putative *katG* mutations were found in these isolates. In contrast, all clones from 2014 had either the *katG* G273R or the G249del mutation fixed and no other alternative candidate mutation at a frequency of ≥0.1%, as revealed by amplicon sequencing (Figure 2). All of these clones were highly resistant to INH (MIC, > 32 μg/mL), based on the resazurin microtiter assay. In addition, clone haplotype analysis established a link between specific *ahpC* mutations and the 2 specific *katG* variants (Figure 2).



B

Sample	G1480	G1479	G516	G520	G249	G252	G535	G841	G842	G993	G1003	G1257	G1478	G1720	G1721	G1928
Date	27/04/09	20/09/13	21/01/14	17/03/14	27/06/14	11/08/14	13/10/14	09/12/14	27/01/15	15/04/15	11/06/15	09/11/15	11/12/15	02/06/16	14/10/16	09/01/17
Sputum/ Culture	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Hospital DST (RIF/INH)[a]	−/−	−/−	−/−	−/−	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/+	−/+	−/−	−/+	−/+
Genomic resistance (RIF/INH)[b]	−/−	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Treatment	RIF,INH, EMB,PZA	RIF,INH, EMB,PZA	RIF,INH, EMB,PZA	RIF,INH, EMB,PZA	RIF,INH, EMB,PZA	RIF,INH, EMB,PZA	RIF,INH, EMB,PZA	RIF,INH, EMB,PZA	RIF,INH, EMB,PZA	RIF,INH, EMB,PZA	RIF,INH, EMB,PZA	RIF,INH, EMB,PZACM,PZA,INH+	RIF,MFX, LZD,INH+	AMK, PZA, LZD,INH+	AMK, PZA, LZD,INH+	AMK, PZA, LZD,INH+
Resistance Type[c]	DS	RR	MDR	MDR	MDR	MDR	MDR	MDR	MDR	MDR	MDR	MDR	MDR	XDR	XDR	XDR

Figure 1. *A*, All drug resistance mutations detected during treatment. Grey scales and symbols indicate drug resistance variants associated with different genes. Solid lines denote mutations detected at a frequency of 100% in the patient's last mycobacteria-positive culture, whereas dashed lines indicate transitory variants over time. *B*, Relevant clinical findings of the case study, including antibiotic therapy, sputum and culture status, results of drug susceptibility testing (DST) in the hospital, and predicted whole-genome sequencing–based resistance profile. Plus signs denote that higher doses of isoniazid were added. AMK, amikacin; CM, capreomycin; DS, drug susceptible; EMB, ethambutol; INH, isoniazid; LZD, linezolid; MDR, multidrug-resistant; MFX, moxifloxacin; PZA, pyrazinamide; RIF, rifampicin; RR, rifampicin resistant; XDR, extensively drug-resistant. [a]Phenotypic analysis was performed using the Bactec-MGIT 960 system (Becton Dickinson, Franklin Lakes, NJ), and genotypic analysis was performed using the GenoType MTBDR plus system (Hain Lifescience, Nehren, Germany). [b]Predicted DST-based resistance, based on mutations detected in genomic regions associated with RIF and INH resistance. [c]Predicted whole-genome sequencing–based drug susceptibility profile.

Thus, multiple lines of evidence suggested that the 2 *katG* mutations were likely involved in INH resistance: (1) genomic analyses identified the variants as mutually exclusive, suggesting selection for different resistant populations; (2) a single-clone phenotypic assay identified that these mutations were associated with high-level INH resistance; and (3) *ahpC* mutations were associated with noncanonical *katG* mutations (Supplementary Table 3).

Additionally, genomic analysis detected 3 different ethambutol-associated mutations, beginning April 2015, including 2 in the *embB* genomic region (G328Y and M306V) and 1 in the *ubiA* region (G165S; Figure 1).

Prospective Case Management Aided by WGS Data

After validation of RIF and INH resistance, we used WGS as a primary tool for detecting resistance. Given the newly discovered multidrug-resistant tuberculosis profile of the patient and their lack of response to treatment, the clinical team decided to change the drug regimen in December 2015. Moxifloxacin (MFX) and capreomycin were added, and RIF was removed. Despite the patient's adherence to the new treatment regimen, sputum smear results were positive in June 2016, followed by another positive culture result in October 2016. Rapid sequencing of this isolate revealed that it had acquired a related MFX resistance mutation (E540D, in *gyrB*) and a likely capreomycin resistance mutation (L16R, in *tlyA*). In parallel, a microdilution-based assay (the Sensititre MycoTB MIC Plate) confirmed resistance to MFX (MIC, < 4 μg/mL) but revealed amikacin susceptibility (MIC, ≤ 1 μg/mL). Accordingly, drug therapy was adjusted, removing MFX and adding linezolid and amikacin. Unfortunately, bedaquiline and delamanid were not available to the hospital. With this new treatment, the last positive culture result was in February 2017, and after 18 months of culture negativity the patient made a satisfactory recovery. All resistance variants detected are in Supplementary Table 4.

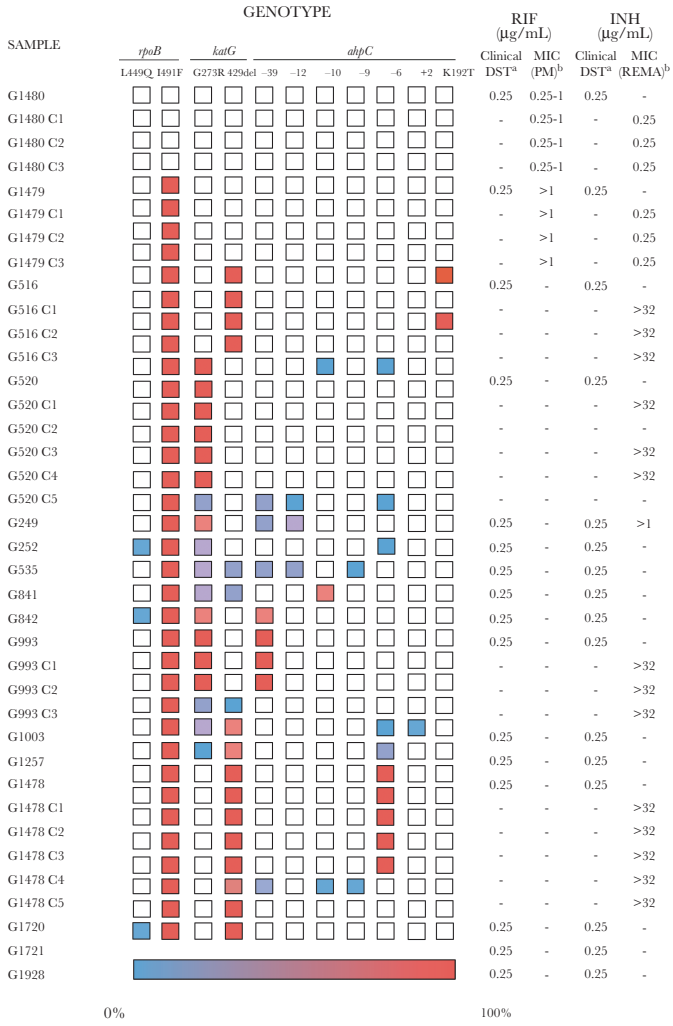


Figure 2. Percentage of the different mutations associated with resistance to isoniazid (INH) and rifampin (RIF). Percentages are given for the frequency among diagnostic cultures, as well as among individual isolated clones (identified with a "C"). DST, drug susceptibility testing; MIC, minimum inhibitory concentration; PM, proportions methods; REMA, resazurin microtiter assay. [a]Phenotypic DST results for RIF and INH from individual clones. [b]Phenotypic DST results for RIF and INH from clinical isolates.

Notably, none of the publically available genomic resistance prediction databases classified any of the isolates as a multidrug-resistant or extensively drug-resistant strain (Supplementary Table 5). In agreement with this, an extensive analysis of 4762 genomes revealed that strains with noncanonical RIF resistance mutations were depleted of known *katG* resistance mutations (7.6% vs 36%; $P < .001$, by the χ^2 test). A deeper analysis of *katG* in those strains revealed 7 mutations not described before, all of them leading to an amino acid change and some with a phylogenetic convergence signal (Supplementary Data).

DISCUSSION

Here we described the use of WGS data to diagnose a case of multidrug-resistant tuberculosis that was missed by the commonly used Bactec-MGIT system. An uncommon RIF resistance mutation (I491F, in *rpoB*) led to a systematic negative test result. Notably, this outcome affected INH DST with the Bactec-MGIT system; in contrast, our investigation clearly demonstrated the presence of high-level INH resistance at different time points. The resistance profile undetected by the Bactec-MGIT system before genomic data were available explains why the patient remained culture positive and the infecting mycobacteria acquired additional resistance mutations between 2013 and 2015. In the absence of a fully reliable Bactec-MGIT result, we decided to use WGS data to aid in the clinical management of the case.

However, clinical decisions based on WGS data are not straightforward. The higher resolution of next-generation sequencing approaches, combined with our increasing knowledge of drug resistance–associated mutations, provide evidence for the usefulness of designing individualized drug regimens [12]. Nevertheless, this work also reveals additional layers of complexity in clinical decision making; for

example, INH-susceptible subpopulations were still present after 2.5 years of treatment (Supplementary Figure 3). These results suggest that personalized treatment will require serial sequencing over time, preferably instead of sputum culture, to avoid culture bias and track the dynamics of susceptible and resistant subpopulations. Furthermore, rigorous standardized statistical approaches such as those developed by Miotto et al [13] should identify highly likely drug resistance mutations, to avoid false-positive predictions and adverse downstream clinical consequences. In this particular case, WGS aided care management, but despite access to WGS data, treatment decisions led to the development of extensively drug-resistant tuberculosis.

The poor treatment outcome in this patient is in line with previous reports that RIF monoresistance is associated with relapse and with the acquisition of additional resistance mutations [2, 14]. Furthermore, it is also noteworthy that most of the variants described are epidemiologically rare and that none of the canonical mutations were found. Uncommon drug resistance–conferring mutations are likely more common in high-burden countries [15], and, thus, personalized treatment approaches based on WGS data in those countries may be compromised. Evidence from this patient adds to the view that we need to integrate different layers of heterogeneity to understand the emergence of and predict drug resistance in a patient. Those layers include strain, lesion, pharmacodynamics, and drug penetration heterogeneity.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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