Drug-Resistant Tuberculosis, Lebanon, 2016 – 2017

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In a 12-month nationwide study on the prevalence of drug-resistant tuberculosis (TB) in Lebanon, we identified 3 multidrug-resistant cases and 3 extensively drug-resistant TB cases in refugees, migrants, and 1 Lebanon resident. Enhanced diagnostics, particularly in major destinations for refugees, asylum seekers, and migrant workers, can inform treatment decisions and may help prevent the spread of drug-resistant TB.

Populations in crisis-affected areas are particularly vulnerable to tuberculosis (TB) linked to malnutrition, overcrowding, and discontinuity in health services (*1,2*). Difficulties accessing diagnosis and starting or completing appropriate treatment can promote the emergence and spread of multidrug-resistant (MDR) TB (resistant to at least rifampin and isoniazid) and extensively drug-resistant (XDR) TB (additionally resistant to ≥1 second-line injectable drug and 1 fluoroquinolone) in the countries of origin or in countries of transit or refuge after migration (*3*).

Lebanon hosts the largest per capita refugee population in the world. In addition to 450,000 refugees from Palestine, ≈1.5 million refugees from Syria are scattered in hundreds of informal sites across the nation (*2,4*). Moreover, the country hosts >250,000 migrant domestic workers, mostly originating from regions with high TB incidence rates, such as Ethiopia, Bangladesh, the Philippines, and Sri Lanka (*5*).

The last national survey on the prevalence of drug-resistant TB in Lebanon was performed 15 years ago (*6*), well before the beginning of the Syria crisis in 2011. Even most recent reported MDR TB rates largely relied on estimates rather than on systematic laboratory confirmation (*6*). Second-line drug susceptibility testing (DST) and individualized XDR TB treatments were not available. We report results from a June 2016–May 2017 nationwide study combining extensive phenotypic and molecular testing. This national survey was approved by the ethics committee of the Azm Center for Research in Biotechnology and Its Applications, Lebanese University (document no. CE-EDST-3-2016), authorized by the Lebanese Ministry of Public Health. Informed consent was obtained from the study patients.

The Study

The study included 720 cases of suspected TB, corresponding to all suspected cases reported from June 1, 2016, through May 31, 2017, to the TB centers from the 9 governorates that make up Lebanon's national TB program. After testing of all corresponding microscopy-positive and microscopy-negative samples, 284 were considered confirmed TB cases on the basis of solid (Lowenstein-Jensen [LJ]) or liquid (BBL MGIT Mycobacteria Growth Indicator, BD Diagnostics, http://www.bd.com) culture results or molecular testing results (Xpert MTB/RIF, Cepheid, http://www.cepheid.com). For samples contaminated with blood, Anyplex MTB/NTM Real-time Detection (Seegene, http://www.seegene.com) (Appendix 1, https://wwwnc.cdc.gov/EID/article/25/3/18-1375-App1.pdf) was used. Thirty-four cases could not be subjected to DST because of culture negativity (n = 28), contamination (n = 3), insufficient sample amount for culture (n = 2), or reagent contingencies (n = 1).

Of the 250 remaining patients, 51% (128/250) were men; the mean age was 34 years (Table 1; Appendix 2, https://wwwnc.cdc.gov/EID/article/25/3/18-1375-App2.xlsx). Patients were from Syria (74/250, 29.6%), Lebanon (70/250, 28%), Ethiopia (57/250, 22.8%), Bangladesh (13/250, 5.2%), Palestine (7/250, 2.8%), or other nations (29/250, 11.6%).

Rifampin resistance was detected among 7/250 (2.8%) patients, concordantly with Xpert testing results for all cases (Table 1). We used multivariate logistic regression to test TB history as an independent predictor of rifampin resistance, after adjusting for age, sex, and nationality (Appendix 1). Log-linearity was checked for age. A 2-tailed type I error rate was set at 5%. TB history information was available for 246 (98.4%) patients. The proportion of

Table 1. Details of 250 TB cases with available phenotypic drug susceptibility profiles, Lebanon, 2016–2017*

Characteristic	No. (%) patients			
	Total, n = 250	New cases, n = 228	Previously treated or relapsed, n = 18	Missing data, n = 4
Sex				
M	128 (51.2)	112 (49.1)	14 (77.8)	2 (50)
F	122 (48.8)	116 (50.9)	4 (22.2)	2 (50)
Country of origin				
Lebanon	70 (28)	64 (28.1)	4 (22.2)	2 (50)
Syria	74 (29.6)	65 (28.5)	9 (50)	0
Ethiopia	57 (22.8)	54 (23.7)	1 (5.6)	2 (50)
Bangladesh	13 (5.2)	13 (5.7)	0	0
Palestine	7 (2.8)	7 (3.1)	0	0
Other	29 (11.6)	25 (11)	4 (22.2)	0
Age, y	34 ± 14	34 ± 14	38 ± 13	23 ± 5
Drug resistance				
RIF	7 (2.8)	3 (1.3)	4 (22.2)	0
Mono	1 (0.4)	1 (0.4)	0	
MDR	3 (1.2)	1† (0.4)	2‡ (11.1)	
XDR	3§ (1.2)	1§ (0.4)	2§ (11.1)	
INH	16 (6.4)	15 (6.6)	1 (5.6)	0
Mono	9 (3.6)	8 (3.5)	1 (5.6)	
INH + SM	7 (2.8)	7 (3.1)	0	
EMB only	1 (0.4)	1 (0.4)	0	0
SM only	23 (9.2)	21 (9.2)	2 (11.1)	0
Susceptible to all first-line drugs	203 (81.2)	188 (82.4)	11 (61.1)	4 (100)

*Age is expressed as mean ±SD; categorical variables are presented as absolute numbers and percentages. EMB, ethambutol; INH, isoniazid; MDR, multidrug resistant; mono, monoresistant; RIF, rifampin; SM, streptomycin; XDR, extensively drug resistant.
†Resistant to RIF and INH.
‡Resistant to RIF, INH, EMB, and SM.
§Resistant to RIF, INH, EMB, SM, amikacin and kanamycin, and levofloxacin (representing all tested drugs for MDR and XDR isolates).

rifampin resistance was 22.2% (4/18) among previously treated patients and patients with relapse and 1.3% (3/228) among patients with new TB cases (adjusted OR 21.4, 95% CI 4.4–105.2; p<0.01). One case in a patient without previous TB history was confirmed by liquid culturing DST as monoresistant to rifampin; 3 other cases, including 1 in a patient without previous TB history, were MDR TB, 2 of which showed resistance to all 4 first-line drugs tested (i.e., ethambutol and streptomycin in addition to rifampin and isoniazid). Moreover, 3 XDR TB cases were detected, including 1 in a patient without previous TB history, showing phenotypic resistance to amikacin, kanamycin, and levofloxacin in addition to all 4 first-line drugs tested. Among all 250 cases, 203 (81.2%) were susceptible to all 4 first-line drugs, 9 (3.6%) were resistant to isoniazid only, 1 (0.4%) to ethambutol only, 23 (9.2%) to streptomycin only, and 7 (2.8%) to isoniazid and streptomycin (Table 1).

To assess their extensive drug-resistance profiles, we subjected isolates from the 3 patients with XDR TB to targeted sequencing by use of a new assay, Deeplex-MycTB (GenoScreen, https://www.genoscreen.fr), which covers 18 drug resistance–associated gene targets (7) (Figure; Appendix 1). Two of these cases were confirmed by whole-genome sequencing. In 1 case (patient identification no. 74), no mutation was found to explain phenotypic resistance to amikacin and kanamycin. For the other drugs for this isolate, and for the 2 isolates analyzed by both tests, we detected drug resistance–associated mutations (8–10)

in *rpoB*, *katG* or *inhA*, *gyrA*, *rrs* or *tlyA*, and *embB*, confirming the resistance phenotypes (Table 2). Moreover, we detected different drug resistance–associated deletions in *ethA* in all 3 XDR TB isolates and drug resistance–associated mutations in *pncA* in 2 XDR TB isolates. These mutations predict additional resistance to ethionamide and pyrazinamide, which are not phenotypically tested in Lebanon or in many other countries.

Results of genotypic analysis of the 3 MDR TB isolates by Deeplex-MycTB also were consistent with phenotypic profiling overall, considering that a rare F129S mutation in *katG* was previously described in association with isoniazid resistance (11), along with other well-established mutations. An ethambutol resistance–associated M306V mutation in *embB* in 1 isolate was phenotypically undetected, probably reflecting known poor phenotypic reproducibility for this mutation (10). Of note, in the same isolate, Deeplex-MycTB testing detected a *gyrA* S91P mutation, which generally confers low levels of levofloxacin resistance (12), as a minority population (5.2%). This detection was confirmed by Anyplex results but was not correlated with phenotypic resistance to levofloxacin tested at a standard critical concentration of 1.5 µg/mL. As with the XDR TB isolates, nonsense insertion or deletion mutations additionally detected in *pncA* or *ethA* predicted supplementary pyrazinamide and ethionamide resistance in some isolates.

None of the MDR or XDR TB cases clustered with any other case in the study population tested by standard

Table 2. Genotypic and phenotypic drug susceptibility profiles of drug-resistant TB cases, Lebanon*

Category			Drug resistance classification					
			MDR			XDR		
Patient ID			14	48	125†	74	168	185
TB drug								
RIF	Gene	rpoB	S450L	S450L	S450L	S450L	**S450L**	**S450L**
	Phenotypic		black	black	black	black	black	black
INH	Genes	katG	S315T	F129S‡	S315T	S315T		**S315T**
		inhA					**C-15T**	
	Phenotypic		black	black	black	black	black	black
PZA	Gene	pncA		Y103Stop	InserA192	A46P		**H71Q**
	Phenotypic		ND	ND	ND	ND	ND	ND
EMB	Gene	embB	Q497R	M306V	M306V	Q497R	**Q497R**	**M306V**
	Phenotypic		black	gray	black	black	black	black
SM	Genes	rpsL	K43R		K88R	K43R		**K43R**
		rrs					**A908C**	
	Phenotypic		black	gray	black	black	black	black
AMI/KAN	Gene	rrs					**A1401G**	**A1401G**
	Phenotypic		gray	gray	gray	black	black	black
FQ	Gene	gyrA		S91P§		D94A	**S91P**	**D94Y**
	Phenotypic		gray	gray	gray	black	black	black
CAP	Genes	tlyA				InserC313		
		rrs					**A1401G**	**A1401G**
	Phenotypic		ND	ND	ND	ND	ND	ND
ETH	Genes	ethA	Deleted¶		141 nt del#	Deleted¶	**DelG632**	**DelG1338**
		inhA					**C-15T**	
	Phenotypic		ND	ND	ND	ND	ND	ND
MIRU-VNTR type**			100-32	19431-157	21404-32	10156-32	21416-15	?-15
M. tuberculosis complex lineage††			2 (Beijing)	4 (Euro-American)	3 (Delhi-CAS)	2 (Beijing)	4 (H37Rv-like)	4 (Haarlem)

*Only genes with detected resistance-associated mutations are shown. No mutation was detected in targets associated with linezolid or bedaquiline and clofazimine resistance. Mutations are shown as amino acid changes with the corresponding codon position, nucleotide changes in promoter regions, or inserted or deleted base (inser or del with position in coding sequence) resulting in a frameshift. Bold text shows mutations concordantly detected by whole-genome sequencing and Deeplex-MycTB (GenoScreen, https://www.genoscreen.fr) in samples subjected to both assays. Other mutations are those detected in samples analyzed by Deeplex-MycTB only. Drug resistance predictions are based on reference data from available scientific literature (8–10), and for *pncA* also on data from Yadon et al. (14). Black represents phenotypic resistance to the different drugs and gray represents phenotypic susceptibility. For phenotypic testing, levofloxacin was the only fluoroquinolone tested. AMI, amikacin; CAP, capreomycin; EMB, ethambutol; ETH, ethionamide; FQ, fluoroquinolones; KAN, kanamycin; INH, isoniazid; ND, not done; PZA, pyrazinamide; RIF, rifampin; SM, streptomycin.
†Deeplex-MycTB result obtained on a primary specimen (sputum). The other results were obtained on indirect samples (primary cultures).
‡Mutation described in association with isoniazid resistance once before by Wang et al. (11). This mutation is not detectable by Anyplex testing.
§Detected as a minority variant, at 5.2% in this sample (see text). Percentages of fixation of other mutations within individual samples range from 80.6% to 100%.
¶Putative deletion, as inferred by absence of reads mapped specifically on the corresponding gene target, in contrast to all other, well covered targets.
#Internal deletion, resulting in a frameshift, from gene position 859 to 999.
**According to MIRU-VNTRPlus website (http://www.miru-vntrplus.org) nomenclature (15). For patient 185, a question mark in the genotype reflects the absence of a detectable allele in locus 4052.
††According to Deeplex-MycTB (spoligotyping and phylogenetic SNPs) and MIRU-VNTRPlus identification, confirmed by whole-genome sequencing results, when done.

24-locus mycobacterial interspersed repetitive unit–variable-number tandem-repeat (MIRU-VNTR) typing of isolates, showing no support for drug-resistance transmission (Appendix 2). Consistently, 4 of the 6 cases involved were previously treated, and the 2 new cases were in migrant workers, presumably representing imported cases. Two cases were in Syria refugees; 1 patient with MDR TB had repeated failed treatment in Syria, and 1 XDR TB case was a relapse after patient arrival in Lebanon. Of the other previously treated cases, 2 had Beijing strain genotypes; the isolate from an XDR TB case in a patient originating from eastern Europe differed by a single allele from the 100-32 MIRU-VNTR haplotype and the isolate from an MDR TB case in a patient from Lebanon fully matched the 100-32 MIRU-VNTR haplotype (Table 2). This haplotype represents a major, presumably highly transmissible MDR-associated clonal complex epidemically spreading across Eurasia (13). Although an XDR TB patient of foreign origin returned to his country after diagnosis because of initial unavailability of proper treatment in Lebanon, the 2 other XDR TB patients received treatment and, as of January 2019, responded positively to ongoing treatments, as were the patients treated for MDR TB.

Conclusions
Although the prevalence of rifampin-resistant TB estimated in Lebanon is relatively low (2.8%), identification of XDR TB and MDR TB cases, including TB strains with strong epidemic potential and complex resistance patterns, calls for sustained diagnosis of MDR TB. We recommend that Lebanon test all TB-positive isolates for resistance to first- and second-line drugs, to inform treatment decisions and prevent the spread of drug resistance. Other major destinations for refugees, asylum seekers, and migrant

Figure. Deeplex-MycTB (GenoScreen, https://www. genoscreen.fr) results identifying an extensively drug-resistant genotypic profile in an isolate from a tuberculosis (TB) patient in Lebanon. Results correspond to TB patient no. 185 in Table 2. Target gene regions are grouped within sectors in a circular map according to the drug resistance with which they are associated. Red indicates target regions in which drug resistance–associated mutations are detected (red text around the map), whereas green indicates regions where no mutation or only mutations not associated with resistance (gray text around the map) are detected. Dark green lines above gene names represent the reference sequences with coverage breadth above 95%. Limits of detection (LOD) of potential heteroresistance (reflected by subpopulations of reads bearing a mutation), depending on the coverage depths over individual sequence positions, are indicated by gray (LOD 3%) and orange (variable LOD >3%–80%) above the reference sequences. Information on mycobacterial species identification, based on *hsp65* sequence best match, and genotype of *Mycobacterium tuberculosis* complex strain, based on spoligotype and lineage-defining phylogenetic SNP, are shown in the center of the circle. AMI, amikacin; BDQ, bedaquiline; CAP, capreomycin; CFZ, clofazimine; EMB, ethambutol; ETH, ethionamide; FQ, fluoroquinolones; KAN, kanamycin; LIN, linezolid; INH, isoniazid; PZA, pyrazinamide; RIF, rifampin; SM, streptomycin; SNP, single-nucleotide polymorphism.
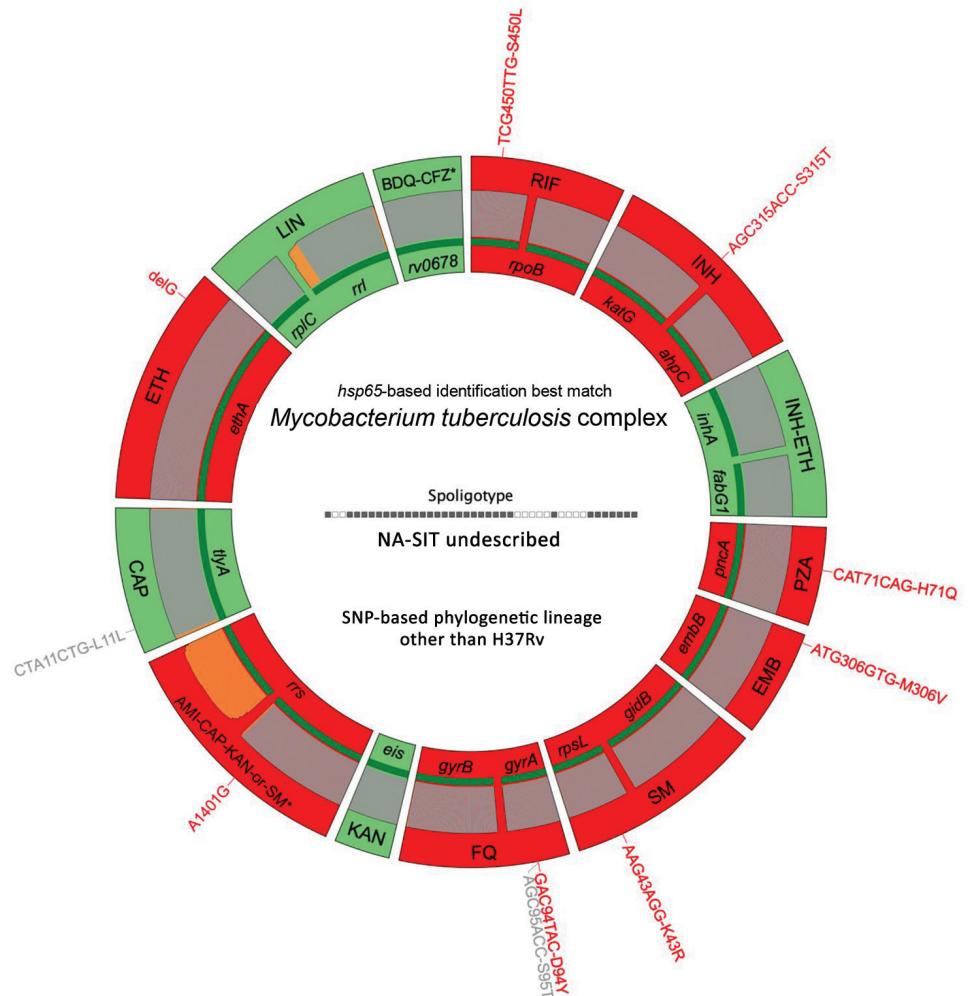
workers should also consider using enhanced diagnostics to help prevent the spread of drug-resistant TB.

About the Author

Ms. El Achkar is a PhD student with supervision shared between Université de Lille and Université Libanaise. Her work focuses on TB drug resistance and transmission in Lebanon.

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Appendix 1

Methods

Patient Data Collection

A standardized form was used to collect patient data, comprising patient age, sex, nationality, TB history, in- or outpatient status, and year of birth. Data were anonymized for subsequent analysis.

Study Design and Population

Clinical samples for all suspected TB cases collected as part of the national TB program from June 1, 2016 through May 31, 2017, were included. Clinical samples from centers located in different governorates in Lebanon, except those in the Northern region, were centralized in the TB reference center in Karentina-Beirut. Then, samples were transferred twice a week, with help from the International Organization for Migration, for subsequent testing to the Laboratoire Microbiologie Santé et Environnement (LMSE) at the Azm Center for Research in Biotechnology and Its Applications at the Lebanese University in Tripoli. Samples from the local TB centers in the Northern region were sent directly to LMSE.

Routine Diagnostics

Confirmation of TB cases was performed in Lebanon as follows. First, microscopic examination was performed in the local TB centers by using Ziehl-Neelsen coloration with a fast cold staining, Kit Quick-TB (RAL diagnostics, http://www.ral-diagnostics.fr), or Cold Kinyoun Stain Kit (Atom Scientific, https://atomscientific.com). Xpert MTB/RIF (Cepheid, http://www.cepheid.com) tests were performed at the Karentina TB center in Beirut or at LMSE in Tripoli.

All samples transferred to LMSE were subjected to microscopic examination or reexamination, and then decontaminated by using standard NaOH/N-acetyl-cysteine treatment.

Primary culture was performed for all microscopy-positive and -negative samples at LMSE by using solid BBL Lowenstein-Jensen LJ (Beckton-Dickinson, https://www.bd.com) and liquid BBL Mycobacteria Growth Indicator Tube (MGIT) (Beckton-Dickinson, https://www.bd.com) media following manufacturer's instructions.

For samples contaminated with blood, samples from paraffin-embedded biopsies, or in case of Xpert-negative and culture-positive results, Anyplex MTB/NTM Real-time detection Kit (Seegene, http://www.seegene.com) was used to confirm identification of *M. tuberculosis* complex.

Phenotypic drug susceptibility testing was performed by using the BD MGIT SIRE (Becton Dickson, https://www.bd.com) liquid culturing kit at critical concentrations for isoniazid (0.1 mg/L), rifampin (1.0 mg/L), streptomycin (1.0 mg/L), or ethambutol (5 mg/L) and the BACTEC MicroMGIT Reader (Becton Dickson, https://www.bd.com) by following the manufacturer's recommendations. In cases of resistance to rifampin and isoniazid, testing was performed by using the same liquid culture system with critical concentrations for amikacin (1.0 mg/L), kanamycin (2.5 mg/L), and levofloxacin (1.5 mg/L) (Sigma Aldrich, https://www.sigmaaldrich.com).

In urgent cases, such as treatment relapse or rifampin resistance detection by Xpert MTB/RIF, Anyplex II MTB/MDR and Anyplex II MTB/XDR detection Kits (Seegene, http://www.seegene.com) were used for detection of mutations associated with isoniazid, rifampin, fluoroquinolones, and injectable drugs.

Whole-genome Sequencing

Whole-genome sequencing was performed at the WHO Collaborating Supranational TB Reference Center at San Raffaele Scientific Institute in Milan, Italy. Genomic DNA was extracted from cultured isolates by using the cetyl-trimethylammonium bromide (CTAB) method (*1*), and quantified by using the Qubit dsDNA BR assay (Life Technologies, ThermoFisher Scientific, https://www.thermofisher.com). Paired-end libraries of 100 bp read length were prepared using the Nextera XT DNA Sample Preparation kit (Illumina Inc., https://www.illumina.com) and sequenced on an Illumina HiSeq 2500 platform according to the manufacturer's instructions. DNA sequence files were deposited in the BioProject database (https://www.ncbi.nlm.nih.gov/bioproject) under accession code PRJNA488372. Downstream

analysis was performed by using a dedicated in-house bioinformatics pipeline in Milan, including quality control check, alignment to H37Rv reference genome, recalibration, and variant calling, as described in Tagliani, et al. (*2*). A mean read coverage depth of >30x, with at least 4 reads on forward and reverse strand, at least 4 allele calls with base quality ≥20, and allele frequency ≥50% were considered acceptable to call variants. The association of mutations with drug resistance was based on available scientific literature (*3–8*).

Targeted deep sequencing

A targeted sequencing approach was used as an alternative molecular test in Lille, France, by using a beta version of Deeplex-MycTB kit (GenoScreen, https://www.genoscreen.fr). Targeted sequencing is more tolerant of small amounts and lower integrity of mycobacterial DNA, which we found in samples transported under suboptimal conditions from Lebanon. Briefly, this assay uses deep sequencing of a single 24-plexed amplicon mix for simultaneous mycobacterial species identification (*hsp*65), genotyping (spoligotyping and phylogenetic SNPs) and prediction of drug resistance of *M. tuberculosis* complex strains. A total of 18 gene regions associated with resistance to first- and second-line drugs are included (Figure). DNA was extracted from heat inactivated clinical specimens or cultured isolates by using MasterPure DNA Purification Kit (Epicenter, Illumina, http://www.epibio.com/). Amplicons were purified by using AMPure XP (Agencourt, Beckman Coulter, https://www.beckmancoulter.com) magnetic beads and quantified by Qubit dsDNA BR assay (Life Technologies, https://www.thermofisher.com). Paired-end libraries of 150 bp read length next-generation sequencing were prepared as described above and sequenced on an Illumina MiSeq platform according to the manufacturer's instructions. DNA sequence files were deposited in the BioProjet database under accession code PRJNA488592. Variant calling and genotypic analysis was performed by using a dedicated, parameterized software developed by GenoScreen (https://www.genoscreen.fr).

MIRU-VNTR typing

Standard 24-locus MIRU-VNTR typing was performed as described in Supply et al. (*9*) by using MIRU-VNTR Typing kits for amplification with 6 quadruplex PCRs (Genoscreen, https://www.genoscreen.fr). The sizes of the amplified fragments and the numbers of repeats in the target loci were determined after capillary electrophoresis-based separation on an ABI 3730 XL DNA Analyzer (ThermoFisher Scientific, Applied BioSystems, www.thermofisher.com),

using a customized software, GeneMapper v.5, (ThermoFisher Scientific, Applied BioSystems, https://www.thermofisher.com). To perform cluster analysis and prediction of genetic lineage of isolates, the genotypes were analyzed and compared with reference strain genotypes by using tools implemented in the MIRU-VNTRplus database (http://www.miru-vntrplus.org), as described in Weniger, et al. (*10*) and Allix-Béguec, et al. (*11*). Genotyping analysis was made blinded from microbiological data and patient data.

Statistical Analysis

Statistical analysis was conducted by using SAS v9.4 software (SAS Institute Inc., https://www.sas.com). Age was expressed as mean ±SD, categorical variables were expressed as absolute numbers and percentages. Multivariate logistic regression was used to test TB history as an independent predictor of drug resistance, after adjustment for age, sex, and nationality (Appendix 2). The log-linearity assumption was checked for the continuous covariate (patient age). The multivariate model was built by first including all predictors and then using a manual backward selection to reduce the model, minimizing Schwarz's Bayesian Criterion and maximizing the c-statistics and the p-value of Hosmer-Lemeshow test. A two-tailed type I error rate of 5% was considered for statistical significance.

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