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Trends of Two Epidemic Multidrug-Resistant Strains of *Mycobacterium tuberculosis* in Argentina Disclosed by Tailored Molecular Strategy

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Abstract. Two *Mycobacterium tuberculosis* strains—M (sublineage 4.1) and Ra (sublineage 4.3)—have long prevailed in Argentina among patients with multidrug-resistant tuberculosis (MDR-TB). Recently, budget constraints have hampered the surveillance of MDR-TB transmission. Based on whole-genome sequence analysis, we used M- and Ra-specific single nucleotide polymorphisms to tailor two multiplex allele-specific polymerase chain reactions (PCRs), which we applied to 252 stored isolates (95% of all newly diagnosed MDR-TB cases countrywide, 2015–2017). Compared with the latest data available (2007–2009), the M strain has receded (80/324 to 20/252, $P < 0.0001$), particularly among cross-border migrants (12/58 to 0/53, $P = 0.0003$) and HIV-infected people (30/97 to 7/74, $P = 0.0007$), but it still accounts for 4/12 new cases of extensively drug-resistant TB. Differently, the Ra strain remained stable in frequency (39/324 to 33/252) and contributed marginally to the extensive drug-resistance load (1/12). Our novel strategy disclosed recent trends of the two major MDR-TB strains, providing meaningful data to allocate control interventions more efficiently.

Multidrug-resistant tuberculosis (MDR-TB)—that is, resistant to at least rifampicin and isoniazid—emerged abruptly in the early 1990s among hospitalized AIDS patients in two overpopulated urban areas of Argentina. At that time, one outbreak strain of MDR *Mycobacterium tuberculosis* was identified in each hot spot.[1,2] Although they rarely crossed the boundaries of their respective areas, these strains—M (single nucleotide polymorphism [SNP] sublineage 4.1, clade Haarlem 2) and Ra (SNP sublineage 4.3, clade LAM 3)—became the most frequent cause of MDR-TB in the country. Today, each one still displays the distinctive core resistance profile of the outbreak onset (strain M has additional resistance to ethambutol, pyrazinamide, and injectable drugs; strain Ra has additional resistance to pyrazinamide). Throughout the 2000s, they still accounted together for 40% of all MDR-TB cases identified countrywide, although their decline had already started to be noticed.[3] We have not performed systematic genotyping since 2009, and our recent efforts to restart MDR-TB transmission surveillance countrywide were inefficient owing to financial and human resource constraints. High-throughput *M. tuberculosis* genotyping is unaffordable for routine use in our reference laboratory. Variable number of tandem repeats-mycobacterial interspersed repetitive units (MIRU-VNTR) typing is performed in the manual version, which is cumbersome and manpower-demanding. By the time we gather a comprehensive MIRU-VNTR dataset for analysis, the data are often outdated.

An alternative approach to monitor the spread of conspicuous strains is to design strain-specific PCRs based on genetic features unique to each one.[4–6] The advent of whole-genome sequencing (WGS) expedited the identification of strain-specific single nucleotide polymorphisms.[7] To gain insight into current trends of strains M and Ra, and to take advantage of the recent sequencing of their genomes,[8,9] we applied a strategy combining the high resolution of WGS with the speed and ease of low-cost PCR-based techniques, an approach that was successful in similar situations.[10–12]

We applied it to characterize the persistence of both strains in terms of 1) frequencies of newly diagnosed MDR-TB cases compared with the latest data available; 2) the presence in vulnerable groups; 3) the presence in the subgroup fitting the definition of extensively drug-resistant TB (XDR-TB), that is, multidrug-resistant tuberculosis with additional resistance to fluoroquinolones and second-line injectables.

Frozen boiled *M. tuberculosis* culture extracts were available for 252 (94.7%) of all 266 patients reported to be newly diagnosed with MDR-TB countrywide in 2015–2017 (one per patient). As a control, we used boiled extracts of 40 isolates (10 M, 10 Ra, and 20 non-M/non-Ra) selected from our frozen collection based on previously determined MIRU-VNTR patterns and spoligotypes.[13]

Whole-genome sequencing (representative isolates of both epidemics covering an ~20-year period, M strain: 262 isolates and Ra strain: 56 isolates) had been performed in previous studies.[8,9] Nucleotide data are deposited in the form of FASTQ reads in the European Nucleotide Archive database (https://www.ebi.ac.uk/ena/browse) under study accession codes PRJEB7669-EMBL-EBI and PRJEB8689-EMBL-EBI.

We designed two multiplex targeted regional allele-specific oligonucleotide PCRs[10] (TRAPs), based on the bioinformatic analysis of their genomes. We tailored one TRAP to target four M strain–specific SNPs and the other to target five Ra strain–specific SNPs. Table 1 describes the characteristics of both assays. The M strain–specific PCR mix contained 1.6 mM $MgCl_2$, 1% dimethyl sulfoxide (DMSO), 0.6 μM M1 primers, 0.5 μM M2 primers, 0.4 μM M3 primers, 0.5 μM M4 primers, 200 mM dNTPs, and 0.05 U/μL recombinant Taq polymerase (Invitrogen, São Paulo, Brazil). Cycling conditions were 95°C for 15 minutes; 27 cycles of 95°C for 1 minute, 64°C for 1 minute, 72°C for 1 minute; and 72°C for 10 minutes. The Ra

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TABLE 1
Characteristics of the multiplex targeted regional allele-specific oligonucleotide PCRs for the M strain and the Ra strain

Assay	SNP	Gene	SNP location	Allele targeted	PCR fragment size (bp)	Primer sequence
M strain	M1	*Rv0101*	112958	Non-M	421	Rv0101F (wt): CCTCTCGCTGCTGGATCTG Rv0101R: CTCCTCGATGGCCCGTTG
	M2	*Rv3915*	4,404,313	M	347	Rv3915F: GATGATCAGCCTGCGCTG Rv3915R (mut): GCCGATCGTTCTTGCCTAAT
	M3	*Rv2220*	2,488,724	Non-M	270	Rv2220F (wt): CTGGAGTTCCGAAGCCCC Rv2220R: GAAACTGATCCACGTCTCGATCA
	M4	*Rv2710*	3,023,036	M	184	Rv2710F (mut): CGCGAAGCCACCGATGAA Rv2710R: GATGACCGCGTTCTCCG
Ra strain	Ra1	*Rv0386*	465768	Ra	447	Rv0386F: TTTGGTGATCGCGCGC Rv0386R (mut): TCACCCTGGTAGGCGAGT
	Ra2	*Rv3830*	4,305,137	Non-Ra	372	Rv3830F (wt): GTCGCTCGGGGAGCTG Rv3830R: CAAGCTGTCCAGGCCTAACT
	Ra3	*Rv2691*	3,009,784	Ra	297	Rv2691F: GACCGAGATGTGCTGCTG Rv2691R (mut): GACGACCTCGGCGACT
	Ra4	*Rv2067*	2,325,706	Non-Ra	210	Rv2067F: CTTGCATAGGCTCGTGGGA Rv2067R (wt): GGCGATCAGAATATCCAGATCG
	Ra5	*Rv1076*	1,201,501	Ra	108	Rv1076F (mut): TATTGCTGCACGACGCG Rv1076R: ACGCCGCAACCTGAAAG

SNP = single nucleotide polymorphism.

strain–specific PCR mix contained 1.6 mM $MgCl_2$, 1% DMSO, 0.5 μM Ra1 primers, 0.3 μM Ra2 primers, 0.3 μM Ra3 primers, 0.2 μM Ra4 primers, 0.5 μM Ra5 primers, 200 mM dNTPs, and 0.05 U/μL recombinant Taq polymerase (Invitrogen). Cycling conditions differed from the M assay only in the annealing temperature (65°C instead of 64°C).

The TRAPs yielded the expected patterns for the 40 control DNAs (Figure 1). Table 2 shows frequencies of both strains in the overall MDR-TB population and in subgroups of interest. Our results disclose the current decline of the M strain, which has been by far the predominant MDR and XDR *M. tuberculosis* strain in Argentina for over 20 years.[3,8] We find it here to be outnumbered by the Ra strain, until recently the second most frequent, which has not receded significantly since 2009, the last surveyed year. Most importantly, the M strain still accounts for one-third of the newly diagnosed XDR-TB cases in the study period.

Overall MDR-TB rates have dropped substantially in most vulnerable groups, particularly among health-care workers, accompanying the decline in the number of health-care workers affected by the M strain. The occurrence of MDR-TB in this vulnerable group is a sharp indicator of flawed hospital infection control interventions; thus, its current decrease may have been fostered by specific control policies which have been in vigor for the last decade, particularly in the hot spot for strain M, that is, implementation of universal TB culture together with rapid methods for drug resistance detection, decentralization of specialized health care, and strengthening of administrative measures for infection control in hospitals with high TB load.[14] Although the numbers of inmates with MDR-TB have decreased, both the M strain and the Ra strain are still lingering in different units of the federal and state prison systems. Regarding the HIV coinfected group, the proportion of MDR-TB patients affected by these epidemic strains has declined. Two decades of universal access of people living with HIV to antiretroviral therapy in Argentina may have contributed to check the spread of both MDR strains by reducing the pool of hypersusceptible hosts.[14,15]

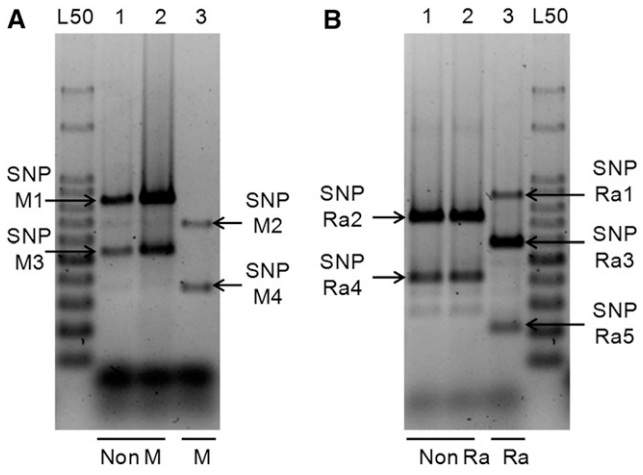

FIGURE 1. Allele-specific multiplex PCR amplification patterns. (**A**) M strain–specific assay. Lane 1 = H37Rv strain; lane 2 = non-M strain; lane 3 = M strain; arrows indicate amplicons corresponding to each of four targeted single nucleotide polymorphisms (SNPs); fragments containing wild-type SNPs M1 and M3 (421 and 270 bp) are amplified by non-M strains; and fragments containing mutated SNPs M2 and M4 (347 and 184 bp) are amplified by the M strain. (**B**) Ra strain–specific. Lane 1 = H37Rv strain; lane 2 = non-Ra strain; lane 3 = Ra strain; arrows indicate amplicons corresponding to each of five targeted SNPs; fragments containing mutated SNPs Ra1, Ra3, and Ra5 (447, 297, and 108 bp) are amplified by the Ra strain; fragments containing wild-type SNPs Ra2 and Ra4 (372 and 210 bp) are amplified by non-Ra strains. L50 = 50-bp ladder.

TABLE 2

Frequencies of new cases of multidrug-resistant tuberculosis caused by two major epidemic strains in various groups of patients in this study, Argentina (2015–2017), compared with last data analyzed countrywide (2007–2009)[3]

Group	M strain no./total no. (%)			Ra strain no./total no. (%)		
	2015–2017	2007–2009	*P*-value	2015–2017	2007–2009	*P*-value*
Newly diagnosed multidrug-resistant tuberculosis	20/252 (7.9)	80/324 (24.7)	< 0.0001	32/252 (12.7)	39/324 (12.0)	0.9110
Migrants	0/53 (0)	12/58 (20.7)	0.0003	0/53 (0)	0/58 (0)	ND
Health-care workers	1/1 (100)	10/13 (76.9)	1.0000	0/1 (0)	0/13 (0)	ND
Inmates†	2/8 (25.0)	11/37 (30.6)	1.0000	3/8 (37.5)	10/37 (27.0)	0.6760
HIV infected‡	7/74 (9.5)	30/97 (30.9)	0.0007	5/74 (6.8)	10/97 (10.3)	0.5868
Newly diagnosed XDR-TB	5/14 (35.7)	4/11 (36.4)	1.0000	1/14 (7.1)	0/11 (0)	1.0000

XDR-TB = extensively drug-resistant tuberculosis; ND = not determined.
* Chi-squared test or Fisher's exact test using MedCalc (https://www.medcalc.org/).
† Prisoners in four different federal or state prisons.
‡ Records on HIV status were available for 58.3% of the patients in the period 2015–2017 and 70.4% of the patients in the period 2007–2009.

The numbers of migrants with MDR-TB have barely changed, although the M strain has receded significantly among them, pinpointing the need to search for additional infection sources. Historically, most migrants come to Argentina from Bolivia and Paraguay, neighboring countries with higher TB, but not MDR-TB, rates. Migrants are not health-screened at arrival but have full access to high-quality health care in our metropolitan reference centers for infectious diseases. Paradoxically, they are at risk of falling ill with the M strain at those very hospitals.[3] Clinical and demographic data in our study show a high input of migrants recently arrived from Peru (31/53) who have developed active MDR-TB in their homeland, where MDR-TB rates are very high.[16]

Two major assets of our study are the completeness of the sample, which includes ~95% of all MDR-TB cases reported countrywide in the period, and the sound microbiological backup provided by our own laboratory, which acts as a reference center for mycobacteria at the national and the supranational level. Two main drawbacks preclude a thorough analysis. First, the genotypes for MDR *M. tuberculosis* strains other than M and Ra are unknown. Second, demographic, epidemiological, and clinical data of the patients are incomplete. In particular, the HIV status was not available for a large proportion of the patients, underlining a persistent gap in information-sharing systems between HIV and TB programs, which surely hinders multidisciplinary and comprehensive programmatic interventions.[17]

By applying a novel strategy that targets the two MDR *M. tuberculosis* strains long prevailing in Argentina, we characterized their current incidences and trends and obtained meaningful information on their spread in vulnerable groups. Certain control issues became exposed which call for programmatic interventions, mainly concerning insufficient data sharing between TB and HIV programs and unidentified current sources of MDR-TB in migrants.

The TRAP approach is not more specific than conventional genotyping and is not meant to replace it but to answer specific questions regarding problematic strains in specific settings. The strategy proves to be transferable and user-friendly. Targeted regional allele-specific oligonucleotide PCRs perform well using boiled crude culture extracts as a source of DNA, requiring neither subculture nor the laborious DNA extraction procedures usually needed for standard genotyping techniques. At our moderate complexity laboratory, TRAPs shorten procedures and reduce costs, thus facilitating the execution of retrospective studies based on frozen culture collections like the one described herein. We carried out the wet laboratory experiments in < 15 workdays at an estimated cost of USD 10 per reaction.

Our TRAPs can also play a role in the clinical setting owing to the distinct drug resistance profiles of the targeted strains. As rapid means to identify strains M and Ra, they would ensure the prompt installation of rational empirical drug schemes while awaiting drug susceptibility testing results. To this end, we are presently evaluating the performance of our TRAPs when applied directly on remnants of those GeneXpert *M. tuberculosis* specimens that are rifampicin resistant, an approach that proved promising in other settings.[18–20]

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