Original Article

Rifampicin Resistance Conferring Mutations among *Mycobacterium tuberculosis* Strains in Rwanda

Isabel Cuella-Martin[1], Jean Claude Semuto Ngabonziza[1,2,3], Gabriela Torrea[1], Conor Joseph Meehan[1,4], Wim Mulders[1], Bertin Ushizimpumu[5], Louise De Weerdt[6], Jelle Keysers[1], Willem Bram De Rijk[1], Tom Decroo[7], Bouke C. De Jong[1], Leen Rigouts[1,8]

[1]Department of Biomedical Sciences, Mycobacteriology Unit, Institute of Tropical Medicine, Departments of [6]Medicine and Health Sciences and [8]Biomedical Sciences, Antwerp University, [7]Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium, [2]Research Innovation and Data Science Division, Rwanda Biomedical Centre, [3]Department of Clinical Biology, University of Rwanda, [5]Department of Biomedical Services, National Reference Laboratory Division, Rwanda Biomedical Center, Kigali, Rwanda, [4]Department of Biosciences, Nottingham Trent University, Nottingham, UK

Abstract

Background: The World Health Organization-endorsed phenotypic and genotypic drug-susceptibility testing (gDST/pDST) assays for the detection of rifampicin-resistant (RR) tuberculosis (TB), may miss some clinically relevant *rpoB* mutants, including borderline mutations and mutations outside the gDST-targeted hotspot region. Sequencing of the full *rpoB* gene is considered the reference standard for rifampicin DST but is rarely available in RR-TB endemic settings and when done indirectly on cultured isolates may not represent the full spectrum of mutations. Hence, in most such settings, the diversity and trends of *rpoB* mutations remain largely unknown. **Methods:** This retrospective study included *rpoB* sequence data from a longitudinal collection of RR-TB isolates in Rwanda across 30 years (1991–2021). **Results:** Of 540 successfully sequenced isolates initially reported as RR-TB, 419 (77.6%) had a confirmed RR conferring mutation. The Ser450 Leu mutation was predominant throughout the study period. The Val170Phe mutation, not covered by rapid gDST assays, was observed in only four patients, three of whom were diagnosed by pDST. Along with the transition from pDST to rapid gDST, borderline RR-associated mutations, particularly Asp435Tyr, were detected more frequently. Borderline mutants were not associated with HIV status but presented lower odds of having *rpoA-C* compensatory mutations than other resistance-conferring mutations. **Conclusion:** Our analysis showed changes in the diversity of RR-TB conferring mutations throughout the study period that coincided with the switch of diagnostic tools to rapid gDST. The study highlights the importance of rapid molecular diagnostics reducing phenotypic bias in the detection of borderline *rpoB* mutations while vigilance for non-rifampicin resistance determinant region mutations is justified in any setting.

Keywords: Borderline mutations, *rpoB*, rifampicin-resistant-conferring mutations, Rwanda, Ser450 Leu

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Introduction

Rifampicin is the core first-line tuberculosis (TB) drug, which is instrumental in achieving a relapse-free cure.[1] Worldwide, there were 450.000 estimated new cases of rifampicin-resistant (RR) TB in 2021, of whom only 32% were diagnosed, reported, and provided with appropriate treatment. The World Health Organization's (WHO's) recommendations concerning treatment for mono-RR do not differ from the multidrug-resistant (MDR)-TB regimen, hence, it is essential to ascertain rifampicin susceptibility to select the correct TB treatment regimen.[2] Culture-based phenotypic drug-susceptibility testing (pDST) methods have been considered the reference standard during the past decades.[3] However, recent studies showed that pDST is not a robust reference for rifampicin-susceptibility testing.[4,5] pDST frequently misses *rpoB* mutations associated with borderline phenotypic resistance, listed as Leu430Pro, Asp435Tyr, His445Asn, His445 Leu, His445Ser, Leu452Pro,

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Address for correspondence: Isabel Cuella-Martin, Department of Biomedical Sciences, Unit of Mycobacteriology, Institute of Tropical Medicine, 155, Nationalestraat, Antwerp 2000, Belgium. E-mail: icuella@itg.be

ORCID:
Isabel Cuella Martin: http://orcid.org/0000-0001-7543-3386

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and Ile491Phe, particularly in liquid-based "rapid" pDST with read-out <2 weeks, such as mycobacterial growth indicator tube 960 media, due to impaired bacterial growth.[4,5] Although these *rpoB* mutations only modestly increase the minimum inhibitory concentration (MIC), rifampicin-based treatment outcomes for patients infected with *Mycobacterium* TB (MTB) bacilli showing borderline resistance to rifampicin is not different from those from patients infected with MTB harboring mutations associated with resistance.[6] Thus, the detection of borderline mutations is as important as that of the other mutations associated with resistance.

Widely employed rapid genotypic DST (gDST) tools, such as GeneXpert MTB/RIF (Xpert; Cepheid, USA) and the GenoType MTBDR*plus* line probe assay (LPA; Hain Life, sciences, Germany), are designed to detect borderline as well as associated RR-conferring mutations in the targeted 81 bp hotspot region of the *rpoB* gene, termed the rifampicin resistance determinant region (RRDR).[7,8] RRDR mutations have been associated with more than 95% of RR-TB, yet with geographical variability.[9,10] However, important non-RRDR mutations are systematically missed by rapid gDST. In some settings, a high prevalence of non-RRDR mutations, such as Ile491Phe, was reported.[9,10] The high rate of this non-RRDR mutation is likely the consequence of diagnostic selective pressure. Xpert and LPA assays have been the global frontline diagnostics for RR-TB, yet they miss Ile491Phe and Val170Phe, another important non-RRDR mutant.[11] Not diagnosed as RR by first-line rapid gDST, patients with non-RRDR RR-TB usually receive repeated rounds of ineffective rifampicin-based treatment, thus allowing undetected mutants to expand clonally. Moreover, the performance of rapid gDST to detect heteroresistance (i.e. mixed population of RR-mutants and wildtype, when the mutants population is still <95%) is variable. Particularly, Xpert performs poorly in samples containing a low proportion of RR mutants.[12]

The use of culture isolates for sequencing the entire *rpoB* gene is considered the reference standard for rifampicin DST.[13] However, sequencing requires high expertise and expensive equipment rarely available in low- and middle-resource settings. Moreover, sequencing results can be biased after repeated cultures as less fit mutants could be lost by outgrowing wild type or more fit mutants.[14]

Since 2005, the programmatic management of drug-resistant TB (PMDT) was launched in Rwanda, and further development resulted in a complete shift from pDST to WHO-recommended rapid molecular diagnostic tools such as Xpert and LPA for initial RR testing, by 2014. The shift to these rapid gDST tools led to a drastic reduction of diagnostic and therapeutic delays, alongside reduced mortality among patients diagnosed with RR-TB.[15] However, this study also showed that HIV-coinfected patients had twice the odds of dying. In Rwanda, while the proportion of HIV-coinfection among all notified TB cases is approximately 21% (National TB Program Rwanda), it accounts for around 40% of RR/MDR-TB patients.[15] To the best of our knowledge, the association between HIV status and borderline mutations is not yet being explored.

Through a longitudinal nationwide collection of RR-TB strains isolated from the pre-PMDT era to 2021, we analyzed the *rpoB* gene sequence data to assess the diversity and trends of RR-conferring mutations in Rwanda as per the diagnostic method used. Moreover, we explored the association between the type of RR-conferring mutations and HIV status and the presence of compensatory mutations in *rpoC* and *rpoA* genes.

Methods

Design and study population

The retrospective analysis included all stored MTB complex (MTBC) isolates from patients diagnosed with RR-TB between 1991 and 2021. The sampling fraction for our study was based on the availability of isolates and it was dependent on the period and coverage [Table 1]. All samples had been collected for diagnostic purposes. The RR-TB patients were diagnosed in three important programmatic phases: (i) before initiation of the PMDT (1991–2005; when no facility was available for DST in Rwanda, sputum specimens were sent to the Institute of Tropical Medicine, Antwerp, Belgium), (ii) early phase of the PMDT (2006–2013; the diagnosis of RR-TB mainly relied on pDST on Löwenstein–Jensen medium [LJ], and later the LPA performed in Rwanda), and (iii) established PMDT (2014–2021; molecular RR-TB diagnostic assays, mainly Xpert, were further expanded to achieve universal DST). Thus, the RR-TB was detected by either LJ-based pDST, LPA, and/or Xpert as previously described.[15]

We extracted *rpoB, rpoA, and rpoC* gene sequence data from whole genome sequencing (WGS) conducted in the context of a separate analysis.[16]

Retrieval of isolates and DNA extraction

The MTBC isolates were retrieved from −80°C freezers and regrown on LJ medium, followed by genomic DNA extraction using an in-house optimized protocol.[17]

Table 1: Sampling fraction of cases used for this study

Year	RR-TB notification*	Included in analysis, *n* (%)
1991–2005	-	114
2006–2013	479	101 (21.1)
2014	73	13 (17.18)
2015	91	34 (37.3)
2016	71	27 (38.0)
2017	56	45 (80.3)
2018	52	33 (63.5)
2019	53	32 (60.4)
2020	38	16 (42.1)
2021	33	4 (12.1)
Total	946	305¥ (32.2)

*Corrected RR-TB based on the false RR finding among samples with very low bacterial load.[18] ¥Exclude 114 isolates collected between 1991 and 2005 as no notification data for that period. RR: Rifampicin-resistant, TB: Tuberculosis

Sequencing and sequence data analysis

WGS of extracted gDNA was outsourced at FISABIO (Valencia, Spain) and KU Leuven (Leuven, Belgium) sequencing facilities. The Illumina HiSeq platform (San Diego, USA) using Nextera XT DNA Library Preparation Kit was performed as previously described.[18] Resistance was confirmed by the detection of all mutations known to be associated with RR or any unknown missense mutation located in the RRDR as directed by the WHO guideline.[19] Mutations were divided, according to the WHO catalog of mutations, into those associated with resistance, associated with resistance-borderline (refer to as borderline), associated with resistance-interim, and uncertain significance.[20] We created a definition of compensatory mutations as mutations in *rpoA* and *rpoC* that have been confirmed as compensatory in the literature.[21]

Metadata collection and analysis

Patients were assigned a unique ID on treatment initiation, and patient files were retrieved from their respective health facilities. The National MDR-TB register and the National Reference Laboratory (NRL) registers were consulted and reviewed to extract relevant data such as demographics, basic clinical presentation, TB treatment history, type of DST used for diagnosis, and result of first RR diagnosis.

To assess the frequency of RR-conferring mutations, these were categorized by year/period of diagnosis or method of diagnosis used. *Z*-test was used to test the difference in proportion.

The association between mutations, HIV coinfection, and the presence of compensatory mutations, was tested in both a univariate and multivariable analysis. In 2005, the PMDT started recording more extensive clinical data. From this period (2005 to 2021), patients with known sex, (estimated) age, province, TB history, and method of diagnosis were included (255/419). Univariate analysis was conducted for each independent variable. Variables with univariate significance or presumed importance based on literature were included in the multivariable analysis. Statistical significance was set at 0.05. R 4.1.0 was used for the logistic regression analysis.

Results

Mycobacterium tuberculosis isolates included

For this analysis, 591 MTBC isolated from patients' samples initially diagnosed with RR-TB from 1991 to 2021 were considered [Figure 1]. Of the 591 RR-TB isolates, 51 (8.6%) presented either poor *rpoB* gene sequence (26, 4.4%) or were contaminated (25, 4.2%). Among 540 successfully sequenced isolates, 419 (77.6%) had at least one RR-conferring mutation in the *rpoB* gene and were therefore included in the analysis, while 121 (22.4%) had a wild-type *rpoB* gene sequence. Of 419, 114 (27.2%) were isolated between 1991 and 2005 (pre-PMDT period), 101 (24.1%) between 2006 and 2013 (early-PMDT period), and 204 (48.7%) between 2014 and 2021 (recent-PMDT period) [Figure 1].

Rifampicin-resistant-conferring *rpoB* mutations

Among 419 strains with RR-conferring mutations, 414 had a single mutation, while 4 had double RR-conferring mutations with some less common mutations at codons 170, 424, 443, and 444 [Table 2]. Five isolates (1.2%) had an RR-conferring mutation outside the RRDR. One carried the Phe424 Leu mutation, diagnosed by pDST, and four carried the Val170Phe mutation. Three Val170Phe mutations were identified among patients diagnosed with pDST during the pre-PMDT period (before 2006). The patient that presented the Val170Phe mutation in heteroresistance with Ser450 Leu was diagnosed in 2014 but the method was not reported.

Among the RRDR mutants, the majority (358; 86.5%) had a single Ser450 Leu mutation, followed by 32 (7.7%) samples with the borderline mutation Asp435Tyr [Table 2]. Of 32 with an Asp435Tyr mutation, 26 (81.3%) were initially detected at diagnosis through Xpert ($n = 22$) or LPA ($n = 4$) but only two by pDST, while the method used for the remaining two was not documented. The relative frequency of Asp435Tyr mutations detected by gDST (13.4%) was significantly higher than by pDST (1.0%) [$Z = 4.914$, $P < 0.0001$, Figure 2].

Throughout the considered period, the Ser450 Leu mutation was predominant, but some diagnostic-driven variability can be observed. Before 2011, when pDST was the only method used for diagnosis, 93.8% of confirmed RR-TB cases had the Ser450 Leu mutation, and the frequency of borderline mutations was lower compared to the gDST testing era (1.9% vs 15.2%). Stratification of all identified RR-conferring mutations by the initial diagnostic tool used, demonstrated a higher mutation variability for gDST used thereafter [Chi-square test: $P < 0.001$, Figure 3].

Factors associated with borderline rifampicin-resistant-conferring mutations

Of 255 patients with complete clinical records, 101 (39.6%) were HIV-coinfected, 165 (64.7%) were men and 128 (50.2%) were from Kigali area. Overall, the mean age was 36.1 (standard deviation = 12.3) with 56 (22.0%) patients over 44 years. Of the 255 patients, 109 (42.7%) were retreatment cases with 63 (57.8%) from the early PDMT period [Table 3]. Borderline mutations accounted for 10.7% of cases among HIV-negative and 15.2% among HIV-coinfected patients. Our multivariable analysis did not find any significant association between the presence of borderline mutations and HIV status.

The univariate analysis showed a statistically significant association between the diagnostic method and the mutation group (odds ratio [OR]: 10.48; 95% confidence interval [CI]: 1.40–78.57, $P = 0.022$), However, in the multivariable analysis, this association was no longer significant (OR: 1.18; 95% CI: 0.07–19.98, $P = 0.990$), after adjusting for other variables.

Among isolates harboring associated with resistance-conferring mutations, 94.2% also presented *rpoA-C* mutations, while only 19.4% of the isolates with borderline mutations exhibited compensatory mutations in *rpoA-C*. The presence of a

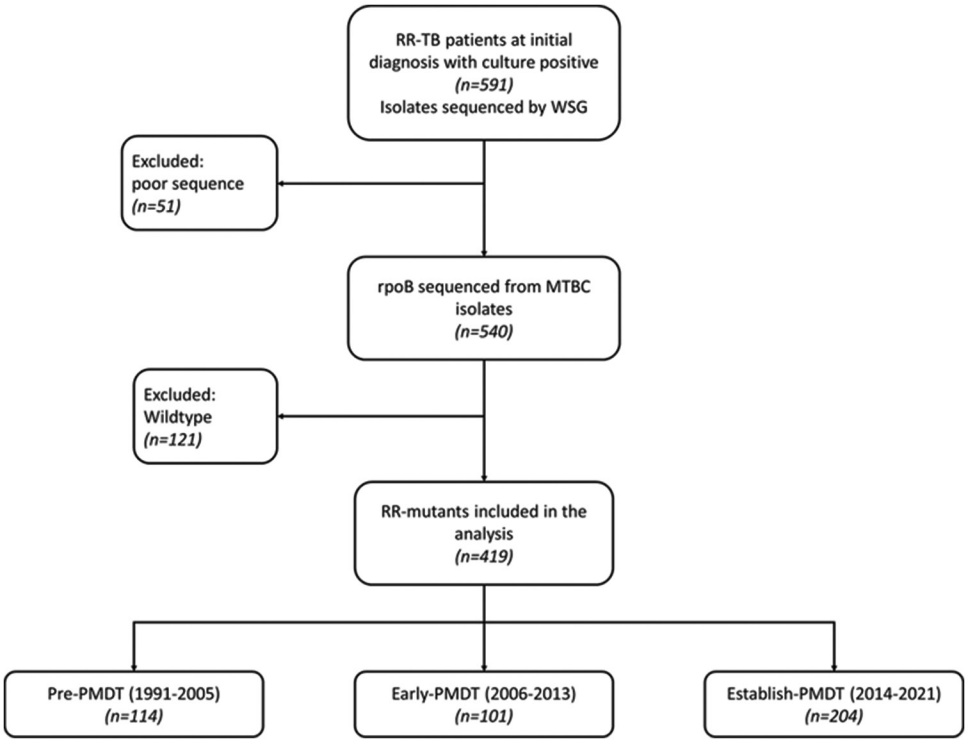

Figure 1: Study participants inclusion flowchart. RR-TB: Rifampicin-resistant tuberculosis, MTBC: *Mycobacterium tuberculosis* complex, PMDT: Programmatic management of drug-resistant TB

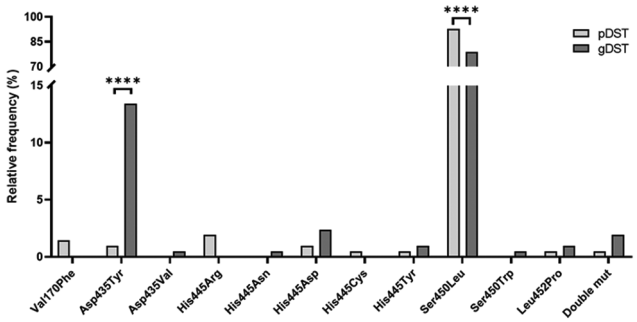

Figure 2: The frequency of rifampicin-resistance-conferring *rpoB* mutations stratified by the initial rifampicin-resistance diagnosis method. pDST: Phenotypic drug-susceptibility testing; gDST: Genotypic drug-susceptibility testing. ****$P < 0.0001$

compensatory mutation in *rpoA-C* was strongly associated with resistance mutation strains (OR: 0.01; 95% CI: 0.00–0.09, $P \leq 0.001$).

Discussion

This nationwide analysis of RR-conferring mutations among MTB strains in Rwanda over three decades showed a predominance of the Ser450 Leu mutation throughout, while borderline mutations, particularly Asp435Tyr, were more frequently identified in recent years when screening for RR-TB was extended to all TB patients, using molecular tools.

Ser450 Leu is indeed the most commonly reported RR-conferring mutation worldwide,[22-24] and has the smallest associated fitness cost compared to other RR-conferring *rpoB* mutants,[25] thus potentially being more transmissible.[23] Indeed, a previous analysis showed a single clone, the "Rwanda RR" clone (R3clone), to cause the majority of MDR-TB disease in which resistance to rifampicin was conferred by the Ser450 Leu.[16] All R3clone isolates have in addition a *rpoC* Pro481Thr mutation potentially involved in restoring its original fitness.[16] This clone is estimated to have arisen in 1987 with an exponential increase in population thought the 1990s, which may explain why the relative frequency of rifampicin-resistance-conferring S450 L mutation steadily increased during the early 90s.

Although seemingly rare, the non-RRDR, Val170Phe mutation was detected among our RR-TB population, most of them in the pre-PMDT period. The Val170Phe mutation is associated with a high rifampicin MIC,[26] and thus easily detectable by pDST, as was the case in our study. As in Rwanda, only previously treated TB patients benefit from pDST also if Xpert shows RS, some patients with such non-RRDR mutation will likely experience at least one unsuccessful round of rifampicin-based treatment before being detected as RR and switched to appropriate MDR-TB treatment. We showed that delays in switching to appropriate MDR-TB treatment were associated with mortality.[15] Since 2009, rifampicin resistance diagnostics used in Rwanda no longer detect the Val170Phe, and consequently, its prevalence is likely underestimated. Similarly, in South Africa and Eswatini diagnostic selective pressure resulted in an increasing prevalence of the Ile491Phe mutation, another important non-RRDR.[9,10] The integration

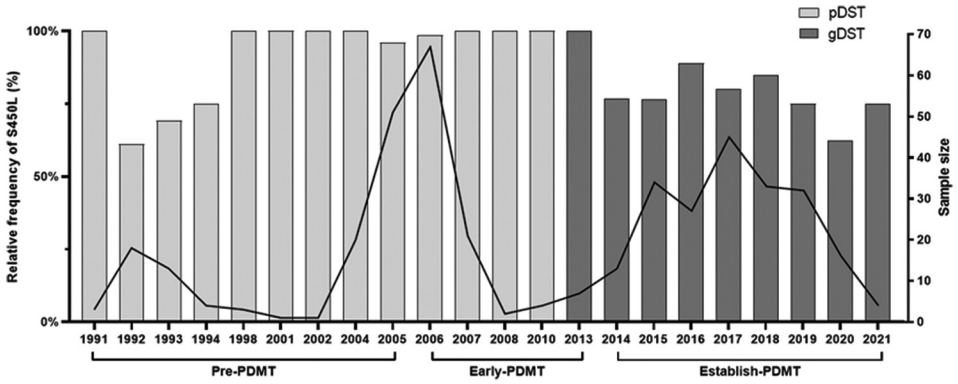

Figure 3: Relative frequency of the rifampicin-resistance-conferring Ser450Leu mutation stratified by time. The bars represent the relative frequency whereas the line describes the sample size per year. Only years with data are displayed. pDST: Phenotypic drug-susceptibility testing; gDST: Genotypic drug-susceptibility testing

Table 2: Rifampicin-resistance-conferring mutations occurring in new and previously treated patients included in the analysis

Mutation	Confidence grading	New	Previously treated	Unknown	Total
Val170Phe	Assoc w R	-	1	2	3
Phe424Leu + Leu430Pro	Uncertain significance Assoc w R-borderline	-	1	-	1
Asp435Tyr	Assoc w R-borderline	26	4	2	32
Asp435Tyr + Ser441Leu	Assoc w R-borderline Assoc w R	1	-	-	1
Asp435Val	Assoc w R	1	-	-	1
Leu443Trp + Thr444Pro	Assoc w R-interim Uncertain significance	1	-	-	1
His445Cys	Assoc w R	-	-	1	1
His445Asp	Assoc w R	3	3	1	7
His445Asn	Assoc w R-borderline	-	1	-	1
His445Arg	Assoc w R	-	-	4	4
His445Tyr	Assoc w R	-	2	1	3
Ser450Leu	Assoc w R	111	165	82	358
Ser450Leu + Val170Phe	Assoc w R Assoc w R	-	-	1	1
Ser450Leu + Ser441Leu	Assoc w R Assoc w R	1	-	-	1
Ser450Trp	Assoc w R	-	1	-	1
Leu452Pro	Assoc w R-borderline	2	-	1	3
Total, *n* (%)		146 (34.8)	178 (42.5)	95 (22.7)	419 (100.0)

Assoc w R: Associated with resistance, Assoc w R-borderline: Associated with resistance-borderline (refer to as borderline), Assoc w R-interim: Associated with resistance

of sequencing-based tools targeting the entire *rpoB* gene and performed directly on sputum in the routine susceptibility testing algorithm would allow earlier detection of important non-RRDR mutations,[27] albeit at a higher cost and complex logistics. Another alternative is direct pDST using thin layer agar (TLA), which is rapid and relatively affordable. The TLA technique showed promising results in Eswatini, also detecting the Ile491Phe mutation.[28] It has recently successfully been implemented in the NRL in Kigali with the aim to expand to peripheral laboratories, to ease its access.

The low proportion of borderline mutations during the pDST period could potentially be explained by operational changes over time. During the pDST era, only retreatment patients were eligible for DST. As rapid molecular assays were rolled out, also the testing indication was widened to microscopy smear-positive new TB patients and since 2014 as the first diagnostic test in high-risk populations such as persons living with HIV. Indeed, the detection of the Asp435Tyr coincides directly with the expansion of Xpert. A separate study revealed an MDR-TB clone possessing the *rpoB* Asp435Tyr mutation, alongside a-15C > T mutation in the *inhA* promoter region *fabG1,* known to confer resistance to both isoniazid and ethionamide.[16] Comparable to the Suriname clone, which was mostly missed by routinely applied pDST, but detected

Table 3: Outcomes of the univariate and multivariate analysis depicting factors potentially associated with borderline mutations

Parameters	Number of patients by *rpoB* mutation group (%)		Univariate		Multivariable	
	Associated with resistance	Borderline	OR (95% CI)	*P*	AOR (95% CI)	*P*
HIV status						
HIV−	125 (89.3)	15 (10.7)	1	1	1	1
HIV+	84 (84.8)	17 (15.2)	1.48 (0.69–3.20)	0.310	2.15 (0.53–8.84)	0.284
Unknown	15 (93.8)	1 (6.2)	0.55 (0.07–4.51)	0.582	1.32 (0.04–45.58)	0.879
Sex						
Female	82 (91.1)	8 (8.9)	1	1	1	1
Male	142 (86.1)	23 (13.9)	1.60 (0.71–3.88)	0.242	1.51 (0.33–7.02)	0.597
Age						
≤24	37 (97.4)	1 (2.6)	1	1	1	1
25–34	80 (84.2)	15 (15.8)	6.94 (0.88–54.51)	0.065	29.53 (1.12–789.28)	0.043
35–44	57 (87.7)	8 (12.3)	5.20 (0.62–43.25)	0.127	5.72 (0.27–119.05)	0.260
≥45	49 (87.7)	7 (12.3)	5.18 (0.61–43.94)	0.131	13.81 (0.55–345.25)	0.110
Province						
Kigali	108 (84.4)	20 (15.6)	1	1	1	1
East	34 (94.4)	2 (5.6)	0.31 (0.07–1.43)	0.135	0.02 (0.00–0.26)	0.002
North	11 (91.7)	1 (8.3)	0.49 (0.06–4.02)	0.507	10.11 (0.66–155.94)	0.100
South	47 (87.0)	7 (13.0)	0.80 (0.31–2.03)	0.645	2.87 (0.48–17.15)	0.248
West	24 (96.0)	1 (4.0)	0.23 (0.03–1.76)	0.155	0.49 (0.03–9.30)	0.633
TB history						
Re-treatment	103 (94.5)	6 (5.5)	1	1	1	1
New	116 (82.3)	25 (17.7)	3.70 (1.46–9.37)	0.006	4.08 (0.67–25.04)	0.128
Unknown	5 (100.0)	0	1.10e−6 (0–infinity)	0.990	6.09e−09 (0–infinity)	0.993
Diagnostic method						
pDST	58 (98.3)	1 (1.7)	1	1	1	1
gDST	166 (84.7)	30 (15.3)	10.48 (1.40–78.57)	0.022	1.18 (0.07–19.98)	0.990
Compensatory mutation *rpoA-C*						
No	13 (34.2)	25 (65.8)	1	1	1	1
Yes	211 (97.2)	6 (2.8)	0.01 (0.01–0.04)	<0.001	0.01 (0.00–0.09)	<0.001

OR: Odds ratio, CI: Confidence interval, NA: Not applicable, AOR: Adjusted OR, TB: Tuberculosis, gDST: Genotypic drug-susceptibility testing, pDST: Phenotypic drug-susceptibility testing

when Xpert was employed,[29] our findings suggest that the Asp435Tyr Rwanda clone had been circulating before the Xpert era but was likely missed due to false susceptible pDST results.

The multivariate analysis of 255 strains with complete clinical records did not reveal any significant association between borderline mutation and HIV infection. Previous research showed that HIV-coinfected patients are more likely to have RR-TB due to mutants with lower fitness levels, as their weakened immune system facilitates bacterial survival.[30] Also in Southern Africa, the circulation of multiple Ile491Phe clones occurred in the context of high rates of HIV co-infection. We did not find such an association, suggesting that the *in vivo* fitness is less impaired than suggested by their *in vitro* growths. Borderline mutations exhibit the same clinical impact and population distribution as high-level resistance mutations.

Our results also described how strains carrying associated with resistance mutation were more likely to bear a compensatory mutation in *rpoA-C*. This association is present even if we correct for the clonality of the R3 clone. Previous studies have suggested that compensatory mutations in *rpoA-C* are more common in strains with high-level resistance,[31-33] which suggests that different operands of compensation exist in strains with borderline mutations, further supporting the evolutionary advantage of compensatory mutations.

Strengths of our analysis include the 30-year duration to investigate the frequency and trend of RR-conferring mutations among TB patients in Rwanda, increasing the representativeness of our findings for the general RR-TB population trend in Rwanda.

Our study also had some limitations. First, the study population underwent significant changes over time. In the early 1990s, some new cases came from a national population survey for RR-TB. It is crucial to understand the 1990s population in the context of Rwanda's genocide history, including a substantial inflow from various countries around. Until 2013, RR-TB testing was only performed on previously treated patients, resulting in most isolates coming from patients

with treatment failure or relapse. Since 2013, there has been a shift in the diagnosis of RR-TB toward including new TB patients, following the revision of the drug-resistance diagnostic approach, advocating for universal DST. Second, only culture-positive samples were sequenced. Although WGS is considered the gold standard, the need for culture has an associated bias. This might slightly affect our trends as the frequency of specific *rpoB* mutations may be underestimated. Moreover, some selection bias may have occurred, as the RR-TB detection rate increased over time when better diagnostic tools became available. Hence, the impact of changes in diagnostic techniques, and indications challenges firm epidemiological conclusions.

In conclusion, our study provides important insights into the variability of RR-TB-conferring mutations observed in Rwanda. We found that the type and frequency of RR-conferring mutations are strongly influenced by the diagnostic method used; emphasizing the need to consider these factors in RR-TB control programs. Our analysis also highlights the rising concern about non-RRDR mutations that may be currently spreading unnoticed. Moreover, we found a significant but probably still incomplete population of RR-conferring mutations in Rwanda over the past 30 years, with Ser450 Leu being the main predominant mutation, but with an increasing representation of borderline mutations. Our data showed how borderline mutations appear to be as clinically relevant as others associated with resistance mutations. Finally, our study underscores the importance of reducing culture bias to mitigate the limitations that may lead to underestimating borderline RR mutations.

Ethical statement

The study protocol was approved by the Rwanda National Ethical Committee, Kigali, Rwanda (IRB 00001497 of IORG0001100; Ref No·0069/RNEC/2017), the Institutional Review Board of the Institute of Tropical Medicine, Antwerp, Belgium (IRB/AB/AC/062; Ref No. 1208/17; May 19, 2018), and the Ethics Committee of the Antwerp University Hospital, Universitair Ziekenhuis Antwerpen Ethische Commissie, Antwerp, Belgium (REG No. B300201836458; May 14, 2018).

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Conflicts of interest

There are no conflicts of interest.

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