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Short Communication

Undetected rifampicin-resistant tuberculosis associated with *rpoB* I491F and V170F mutations in Botswana: Diagnostic implications

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ABSTRACT

Background: Undetected rifampicin resistance is a threat to global tuberculosis (TB) control efforts by delaying effective treatment. In different studies, non-canonical *rpoB* mutations outside the rifampicin resistance-determining region have been reported at varying prevalences by country. Here, we report cases of rifampicin resistance in Botswana that were missed by the routine molecular diagnostic assays.
Methods: Individuals were tested under routine programme conditions, in accordance with national guidelines, at four designated drug-resistant TB clinics from 2017 to 2022. Initial testing at the facilities included GeneXpert MTB/RIF ultra and later phenotypic drug susceptibility testing (pDST), as well as the Hain MTBDR*sl* line probe assay, at the National Tuberculosis Reference Laboratory. A total of nine isolates were subsequently sequenced on the Illumina NextSeq 2000 instrument.
Results: At the point of care, routine molecular tests classified all nine individuals as susceptible to rifampicin. Subsequent culture and phenotypic drug susceptibility testing confirmed rifampicin resistance. Whole-genome sequencing identified non-canonical *rpoB* mutations outside the rifampicin resistance-determining region I49F and V170F, which are associated with low-level rifampicin resistance. Of the nine isolates sequenced, 4 (44%) harboured the *rpoB* V170F mutation, while 5 (56%) harboured the *rpoB* I491F mutation.
Conclusions: These results highlight a diagnostic gap within the current algorithms and show the value of sequencing-based approaches for accurately detecting drug resistance. Incorporating sequencing into routine clinical practice could help guide the selection of TB treatment and improve treatment outcomes in patients who do not respond to first-line therapy.

Introduction

Rifampicin-resistant tuberculosis (RR-TB) continues to be a major global health challenge, particularly in high TB/HIV countries. Many of the World Health Organization (WHO)-endorsed molecular assays, such as the GeneXpert MTB/RIF Ultra (Cepheid, USA), TrueNat MTB/RIF (Molbio diagnostics, India), BD MAX MDR-TB (Becton Dickinson, Franklin Lakes, NJ, USA) Cobas MTB-RIF/INH

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Table 1
Clinical and microbiological characteristics of patients whose *Mycobacterium tuberculosis* strains harbouring *rpoB* I491F and V170F mutations.

Sample ID	Gender	Age	Health district	HIV status	*rpoB* mutation	Prior TB treatment	Sublineage
BTB019	F	43	Greater Gaborone	Positive	V170F	No	lineage1.2.2.2
BTB093	M	34	Greater Francistown	Negative	V170F	No	lineage1.2.2.2
BTB174	M	40	Greater Palapye	Positive	V170F	Yes	lineage1.2.2.2
BTB205	M	35	Greater Gaborone	Negative	V170F	Yes	lineage4.1.1.3
BTB032	F	56	Greater Gaborone	Negative	I491F	Yes	lineage4.3.3
BTB052	F	29	Greater Gaborone	Positive	I491F	No	lineage4.3.3
BTB130	M	44	Greater Gaborone	Positive	I491F	No	lineage4.3.3
BTB131	M	33	Greater Gaborone	Positive	I491F	No	lineage4.3.3
BTB146	F	32	Kgalagadi	Positive	I491F	Yes	lineage4.3.3

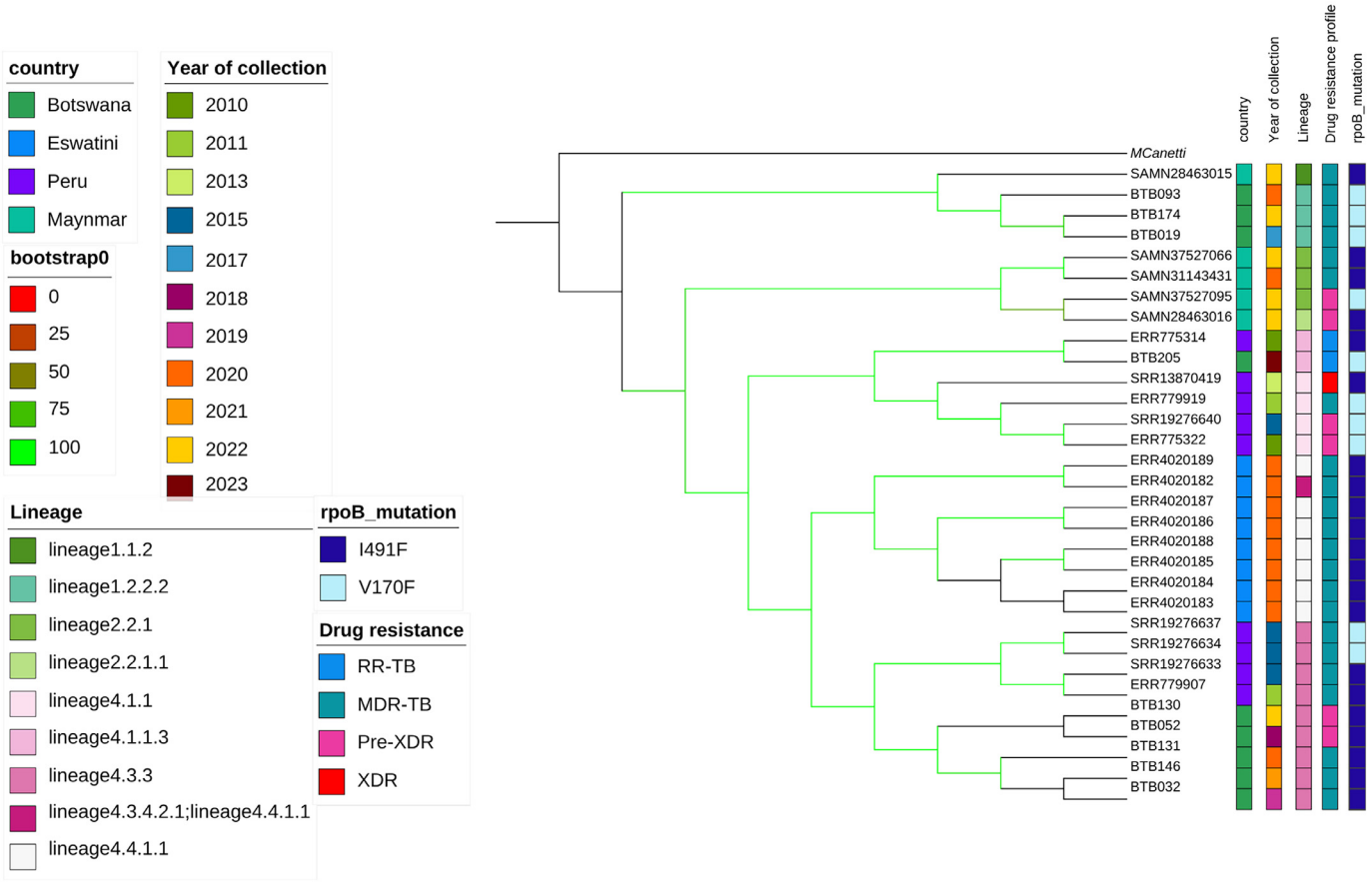

Fig. 1. Maximum-likelihood phylogenetic tree of *Mycobacterium tuberculosis* isolates from Botswana, Eswatini, Peru, and Myanmar. Isolates are annotated by drug resistance profiles, *rpoB* mutations, collection year, and lineage.

(Roche Diagnostics International AG, Rotkreuz, Switzerland), and line probe assays (LPAs) (GenoType MTBDR*plus*, Hain Lifesciences), target the 81-bp resistance-determining region (RRDR) in the *Mycobacterium tuberculosis* (*Mtb*) *rpoB* gene and therefore cannot detect mutations outside this region [1–5]. However, resistance to rifampicin may also occur due to the *rpoB* I491F or V170F mutation outside the RRDR that are known to confer low-level resistance to rifampicin (minimum inhibitory concentration [MIC] of 0.5μg/mL) [6,7]. These are often missed by these molecular assays, particularly when MIC testing or whole genome sequencing (WGS) is not performed [8].

In Botswana, the GeneXpert MTB/RIF Ultra assay is the primary diagnostic tool for detecting *Mtb* and rifampicin resistance. Currently, there is limited data on the prevalence of variants with *rpoB* I491F and V170F mutations in Botswana.

Methods

During routine diagnostic testing for tuberculosis with the GeneXpert MTB/RIF Ultra assay, nine samples obtained from patients in the Gaborone, Francistown, Kgalagadi and Palapye health districts were submitted to the National Tuberculosis Reference laboratory for culture and phenotypic drug susceptibility testing (DST), despite the GeneXpert MTB/RIF test calling the samples susceptible to rifampicin. Samples were further tested for additional resistance with the Hain MTBDR*sl* assay. To determine the mechanism of rifampicin resistance in these isolates, whole-genome sequencing was performed on the Illumina NextSeq 2000 instrument (Illumina, San Diego, CA, USA), and the data were analysed using the MTBseq pipeline in combination with TB-Profiler version 6.2.1, as previously described [9,10]. We used descriptive statistics,

including counts and percentages, to describe the distribution of *rpoB* mutations across the sequenced isolates.

Results

All nine samples were culture positive, and phenotypic testing identified rifampicin resistance. Additionally, the MTBDR*sl* assay detected fluoroquinolone resistance in two isolates, while no resistance was detected for kanamycin, capreomycin, and amikacin. The isolates, cultured between 2017 and 2022, were obtained from patients residing in Greater Gaborone, Greater Francistown, Greater Palapye, and Kgalagadi health districts, indicating a widespread presence of genotypically susceptible, phenotypically resistant *Mtb* strains. Among these, five were newly diagnosed, while four had been previously treated; six were people living with HIV. Sequence analysis revealed that five isolates harboured the *rpoB* I491F mutation and belonged to sublineage 4.3.3. The remaining four isolates harboured the V170F mutation, with three belonging to sublineage 4.1.1.3 and one to sublineage 1.2.2.2 (Table 1)**.** According to the WHO mutation catalogue, all five sublineage 4.3.3 isolates are genotypically resistant to rifampicin, isoniazid, ethambutol, pyrazinamide, and ethionamide [11].

Notably, two of these isolates had variants associated with moxifloxacin and levofloxacin resistance, and were classified as pre-extensively drug-resistant (pre-XDR). The single sublineage 4.1.1.3 isolate, with a *rpoB* V170F mutation, was classified as rifampicin monoresistant, with no additional drug resistance detected. The three sublineage 1.2.2.2 isolates were genotypically resistant to rifampicin, isoniazid, ethambutol, and ethionamide. No variants known to confer resistance to bedaquiline, delamanid, or clofazimine were detected. Our phylogenetic analysis shows that *Mtb* isolates with I491F or V170F mutations are not restricted to a single lineage or geographic region in Botswana. Importantly, the detection of new TB cases confirms the transmission of *Mtb* with either the I491F or V170F *rpoB* mutation.

Previous studies in Botswana, Peru, Eswatini, South Africa, Mozambique, and Myanmar have reported on the presence of *Mtb* isolates with the I491F or V170F mutation [8,12–16]. A phylogenetic reconstruction revealed that isolates from Botswana shared lineage similarity with those from Peru [14] and Myanmar [8], but were unrelated to the lineage identified in Eswatini [13] (Figure 1). The *Mtb* isolates with the I491F mutation reported in Eswatini belong to sublineage 4.4.1.1. We report a sublineage 4.1.1.3 strain with the *rpoB* V170F mutation; the rest of the strains with the I491F belonged to sublineage 4.3.3. A recent study in Botswana also reported a sublineage 4.3.3 variant with *rpoB* I491F mutation [12]. The Eswatini variants with the *rpoB* I491F mutation also harboured the *Rv0678* M146T mutation, which confers resistance to bedaquiline and clofazimine [13]; however, none of the variants detected in Botswana harboured this mutation.

Conclusion

Our findings demonstrate the presence of circulating *Mtb* strains with *rpoB* I491F or V170F mutations, which are currently missed by the implemented molecular diagnostic assays. The true extent of this undetected rifampicin resistance can only be determined through studies with larger samples sizes. Such studies would also enable assessment of the association between these *rpoB* mutations and factors such as HIV status and TB treatment history. Accordingly, we recommend implementing targeted next-generation sequencing using the Deeplex Myc-TB assay (Genoscreen, France) as a rapid reflex test for patients who do not respond to first-line therapy, where rifampicin resistance may be undetected.

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Ethical approval: The study was approved by the Stellenbosch University Health Research Ethics Committee (HREC) (HREC reference no: S22/12/271 [PhD]) and the Ministry of Health Human Research Development Division [reference no: HPRD: 6/14/1]. Permission to use de-identified participant clinical information was granted; therefore, informed consent was waived. This study was conducted in accordance with the guidelines and regulations established by the ethics committees of the respective institutions and the Declaration of Helsinki.

Data availability: The datasets presented in this study are available in online repositories. The data can be found here https://www.ebi.ac.uk/ena/browser/home, accession number PRJEB83872.

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