Outbreak of multidrug-resistant tuberculosis in South Africa undetected by WHO-endorsed commercial tests: an observational study

Ndivhuho A Makhado, Edith Matabane, Mauro Faccin, Claire Pinçon, Agathe Jouet, Fairouz Boutachkourt, Léonie Goeminne, Cyril Gaudin, Gugu Maphalala, Patrick Beckert, Stefan Niemann, Jean-Charles Delvenne, Michel Delmée, Lufuno Razwiedani, Maphoshane Nchabeleng, Philip Supply, Bouke C de Jong*, Emmanuel André**

Summary

Background Global roll-out of rapid molecular assays is revolutionising the diagnosis of rifampicin resistance, predictive of multidrug-resistance, in tuberculosis. However, 30% of the multidrug-resistant (MDR) strains in an eSwatini study harboured the Ile491Phe mutation in the *rpoB* gene, which is associated with poor rifampicin-based treatment outcomes but is missed by commercial molecular assays or scored as susceptible by phenotypic drug-susceptibility testing deployed in South Africa. We evaluated the presence of Ile491Phe among South African tuberculosis isolates reported as isoniazid-monoresistant according to current national testing algorithms.

Methods We screened records of 37 644 *Mycobacterium tuberculosis* positive cultures from four South African provinces, diagnosed at the National Health Laboratory Service–Dr George Mukhari Tertiary Laboratory, to identify isolates with rifampicin sensitivity and isoniazid resistance according to Xpert MTB/RIF, GenoType MTBDR*plus*, and BACTEC MGIT 960. Of 1823 isolates that met these criteria, 277 were randomly selected and screened for Ile491Phe with multiplex allele-specific PCR and Sanger sequencing of *rpoB*. Ile491Phe-positive strains (as well as 17 Ile491Phe-bearing isolates from the eSwatini study) were then tested by Deeplex-MycTB deep sequencing and whole-genome sequencing to evaluate their patterns of extensive resistance, transmission, and evolution.

Findings Ile491Phe was identified in 37 (15%) of 249 samples with valid multiplex allele-specific PCR and sequencing results, thus reclassifying them as MDR. All 37 isolates were additionally identified as genotypically resistant to all first-line drugs by Deeplex-MycTB. Six of the South African isolates harboured four distinct mutations potentially associated with decreased bedaquiline sensitivity. Consistent with Deeplex-MycTB genotypic profiles, whole-genome sequencing revealed concurrent silent spread in South Africa of a MDR tuberculosis strain lineage extending from the eSwatini outbreak and at least another independently emerged Ile491Phe-bearing lineage. Whole-genome sequencing further suggested acquisition of mechanisms compensating for the Ile491Phe fitness cost, and of additional bedaquiline resistance following the introduction of this drug in South Africa.

Interpretation A substantial number of MDR tuberculosis cases harbouring the Ile491Phe mutation in the *rpoB* gene in South Africa are missed by current diagnostic strategies, resulting in ineffective first-line treatment, continued amplification of drug resistance, and concurrent silent spread in the community.

Funding VLIR-UOS, National Research Foundation (South Africa), and INNOVIRIS.

Introduction

Rapid diagnosis of *Mycobacterium tuberculosis* resistant to anti-tuberculosis drugs is essential to prevent further acquisition of drug resistance, high morbidity and mortality, and unabated transmission of resistant strains. Fast detection of resistance to rifampicin, the most important anti-tuberculosis drug, is especially crucial because it is predictive of multidrug resistance, defined as resistance to at least isoniazid and rifampicin.

South Africa has been a global leader in nationally deploying two rapid molecular assays endorsed by WHO: GenoType MTBDR*plus* version 2.0 (Hain Lifescience, Nehren, Germany) and Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA). Since 2012, the Xpert MTB/RIF assay has progressively replaced smear microscopy as the first-line test to rapidly diagnose tuberculosis and rifampicin resistance among people with presumptive tuberculosis in South Africa. These tests are complemented by culturing with the BACTEC mycobacteria growth indicator tube (MGIT) 960 automated detection system (BD, Franklin Lakes, NJ, USA), introduced as the gold standard for drug-susceptibility testing in South Africa in the early 2000s.

However, these tests, similarly to the most recently developed Xpert MTB/RIF Ultra (Cepheid) and NTM+MDRTB (Nipro, Osaka, Japan) assays,[1,2] by design detect only mutations located within the so-called rifampicin resistance-determining region (RRDR) of the *rpoB* gene, covering more than 95% of known

Lancet Infect Dis 2018

Published **Online** October 17, 2018 http://dx.doi.org/10.1016/S1473-3099(18)30496-1

See **Online/Comment** http://dx.doi.org/10.1016/S1473-3099(18)30550-4

*Joint senior authors

Department of Medical Microbiology, National Health Laboratory Service–Dr George Mukhari Tertiary Laboratory, Pretoria, South Africa (N A Makhado MSc, M Nchabeleng MD); **Department of Microbiological Pathology, Sefako Makgatho Health Sciences University, Pretoria, South Africa** (N A Makhado, E Matabane MSc, M Nchabeleng); **Mycobacteriology Unit, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium** (N A Makhado, B C de Jong MD); **Global Health Institute, University of Antwerp, Belgium** (N A Makhado); **Institute of Information and Communication Technologies, Electronics and Applied Mathematics, University of Louvain, Brussels, Belgium** (M Faccin PhD, J-C Delvenne PhD); **Centre Hospitalier Universitaire, Lille, EA 2694–Santé Publique: Épidémiologie et Qualité des Soins, University of Lille, Lille, France** (C Pinçon PhD); **Genoscreen, Lille, France** (A Jouet PhD, C Gaudin PhD); **Medical Microbiology Pole, Institute of Clinical and Experimental Medicine, UC Louvain, Brussels, Belgium** (F Boutachkourt BSc, L Goeminne BSc, M Delmée MD); **National Reference Laboratory, Ministry of Health, Mbabane, eSwatini** (G Maphalala MSc); **Molecular and Experimental Mycobacteriology, Research**

Centre Borstel, Leibniz Lung Centre, Borstel, Germany (P Beckert PhD, S Niemann PhD); **German Centre for Infection Research, Borstel Site, Borstel, Germany** (P Beckert, S Niemann); **Centre for Operations Research and Econometrics, UC Louvain, Louvain-La-Neuve, Belgium, Belgium** (J-C Delvenne); **Department of Community Medicine, Sefako Makgatho Health Sciences University, Pretoria, South Africa** (L Razwiedani MD); **Gauteng Department of Health, Hatfield, South Africa** (L Razwiedani); **Centre National de la Recherche Scientifique, Lille, France** (P Supply, PhD); **Institut National de la Santé et de la Recherche Médicale, Lille, France** (P Supply); **Centre Hospitalier Universitaire de Lille, Lille, France** (P Supply); **University of Lille, Lille, France** (P Supply); **U1019, UMR 8204, Centre for Infection and Immunity of Lille, Institut Pasteur de Lille, Lille, France** (P Supply); **and Laboratory of Clinical Bacteriology and Mycology, Department of Microbiology and Immunology, KU Leuven, Leuven, Belgium** (E André MD)

Correspondence to:
Dr Emmanuel André, Laboratory of Clinical Bacteriology and Mycology, Department of Microbiology and Immunology, KU Leuven, Leuven 3000, Belgium
emmanuel.andre@uzleuven.be

Research in context

Evidence before this study

We searched PubMed for studies published before Jan 1, 2018, that reported on isolates bearing the *rpoB* Ile491phe mutation, which is associated with rifampicin-resistant tuberculosis but missed by WHO-endorsed rapid molecular assays or is scored as susceptible by phenotypic drug susceptibility tests. Different combinations of the following search terms were used: "*rpoB* sequencing", "(Ile)491(Phe)" (*Mycobacterium tuberculosis* numbering system), and "(Ile)572(Phe)" (*Escherichia coli* numbering system). Although the presence of this mutation has not been systematically investigated in many world regions, a number of studies since 1999 have reported its occasional detection in isolates from Australia (from a patient born in Vietnam), Bangladesh, China, Kuwait, Pakistan, occupied Palestinian territory, Syria, Turkey, Mexico, and the UK. As a single notable exception, a study published in 2015 revealed that 30% of multidrug-resistant (MDR) tuberculosis strains from a national survey done in eSwatini in 2009 harboured this mutation. This mutation could not be identified by a drug-resistance survey done in South Africa between 2012 and 2014, which used WHO-endorsed laboratory techniques to screen for rifampicin resistance. Likewise, the frequency of this mutation was not specifically examined in a dataset from a large genomic analysis of more than 5000 isolates from five continents.

Added value of this study

Our study shows that a substantial proportion of patients with MDR tuberculosis in the North West, Gauteng, and Mpumalanga provinces of South Africa are infected by strains that carry the *rpoB* Ile491phe mutation. This mutation arose independently in at least two distinct strain lineages in South Africa, only one of which represents the longitudinal extension of the Ile491phe-related outbreak prevailing in eSwatini. These cases of MDR tuberculosis are thus not detected by the WHO-approved diagnostic algorithm in place in South Africa. As seen from our data, this diagnostic gap results in patients receiving multiple rounds of unsuccessful rifampicin-based treatment, systematic amplification of resistance to at least all first-line drugs, and onward transmission of MDR tuberculosis in the studied settings. In strain subsets, we also found genomic evidence of the acquisition of mechanisms mitigating the fitness cost of drug resistance, and of ongoing emergence of decreased sensitivity to bedaquiline, introduced only recently in the national treatment programme for selected patients with drug-resistant tuberculosis. Upon screening of a recent global dataset of more than 5000 isolates, 20 independent emergence events of strain groups bearing this and other mutations undetected by WHO-endorsed tests could be counted among rifampicin-resistant strains at least in Africa, Asia, and Europe, indicating that this emergence is not restricted to southern Africa.

Implications of all the available evidence

The emergence and longitudinal spread of tuberculosis strains with the *rpoB* Ile491Phe mutation highlight the limits of current WHO-endorsed diagnostic systems for comprehensively detecting rifampicin resistance and subsequently MDR tuberculosis. Even if the prevalence of these mutations were to be globally low at present, strains harbouring this and other mutations undetected by these tests will predictably be selected as a result of their escape from these diagnostics, unless implementation strategies are improved. Our findings thus stress the importance for South Africa, as well as other countries (particularly in southern Africa), to urgently adjust the surveillance and diagnostic algorithms to detect such strains. Failure to do so could lead to more cases resistant to all first-line drugs and more treatment failures among patients thought to have isoniazid-monoresistant tuberculosis, as well as unabated transmission of these strains. Importantly, the recent WHO guidelines for first-line treatment of isoniazid-monoresistant tuberculosis supplemented with fluoroquinolone should be used only in places where the circulation of these MDR tuberculosis strains or individual infections with these strains can be excluded. Missed resistance to rifampicin and (possibly) other first-line drugs, followed by fluoroquinolone enhanced treatment would otherwise be disastrous, as resistance to fluoroquinolones—the key class of drugs for the treatment of MDR tuberculosis—would quickly ensue.

rifampicin-resistance mutations among *M tuberculosis* strains.[3] The tests do not detect rifampicin resistance associated with *rpoB* mutations in codons 170 and 491, located outside the RRDR.[4] Although available data suggest that these and other crucial mutations outside the RRDR might be of low frequency globally,[5] the mutations could be more prevalent regionally.[6,7] Importantly, 30% of the multidrug-resistant (MDR) tuberculosis isolates from a survey done in eSwatini in 2009—before large-scale introduction of the Xpert MTB/RIF test—harboured the Ile491Phe mutation.[8] Furthermore, this mutation and others located inside or outside the RRDR are also routinely identified as sensitive by phenotypic drug-susceptibility testing, including BACTEC MGIT 960.[9,10] The latest national drug resistance survey in South Africa did not include methods able to detect the potential emergence of these difficult-to-diagnose MDR tuberculosis strains.[11]

This diagnostic gap raises concerns about the potential silent spread of MDR tuberculosis strains harbouring such mutations. We therefore evaluated whether MDR tuberculosis strains genotypically similar to those identified in eSwatini were also present in South Africa.

Methods

Study setting

This study was done at the National Health Laboratory Service–Dr George Mukhari Tertiary Laboratory

(NHLS–DGMTL) in Pretoria, South Africa, a high-throughput diagnostic and referral laboratory accredited by the South African National Accreditation Standards. The laboratory receives clinical specimens from the clinics and hospitals in Gauteng province, and from patients referred by peripheral clinics in the North West, Limpopo, and Mpumalanga provinces. The laboratory also serves the Dr George Mukhari Academic Hospital, a 1650-bed hospital partly serving the same four provinces.

The study was approved by the Research Ethics Committee of Medical University of South Africa (now Sefako Makgatho Health Sciences University, Pretoria, South Africa; approval number SMUREC/M/54/2017/PG). The confidentiality of patients' data was maintained throughout the study.

Data entry

Patient demographic data and clinical information collected from the NHLS–DGMTL information system and medical records were double-entered in spreadsheets (Microsoft Excel, version 15 [2013]).

Treatment outcome of the present treatment episode was recorded from routine tuberculosis registers, following WHO definitions: treatment failure was defined as smear positivity after 5 months of treatment, cure was defined as smear negativity after 5 months of treatment, and treatment completed was defined as no known poor outcome after 6 months of treatment without microscopy confirmation at 5 months. In our analysis, the combination of cure and treatment completed was considered as favourable treatment outcome, and the combination of treatment failure, defaulted, and died as unfavourable treatment outcome. Patients with favourable outcome were not systematically followed up for relapse. Patients whose records could not be verified, and those who transferred out or were lost to follow-up, were considered to have an unknown clinical outcome.

Sample selection

Between Jan 1, 2013, and Sept 30, 2016, 217 348 clinical samples from the four provinces were sent to NHLS–DGMTL for diagnosis of tuberculosis. The samples were mainly sputum samples collected by the referring clinics and laboratories, and were stored at 4°C when they could not be sent or processed on the same day. The samples were routinely examined by microscopy, Xpert MTB/RIF, line probe assay (GenoType MTBDR*plus*), and MGIT liquid culturing (BACTEC MGIT 960) first-line drug-susceptibility testing. Isoniazid was tested at a concentration of 0·1 mg/mL and rifampicin at 1·0 mg/mL. 14 067 (6·5%) samples were smear positive, and 37 644 (17·3%) were culture positive. Positive cultures for which no second-line drug-susceptibility testing was required (ie, MDR tuberculosis was absent) were routinely stored at –20°C in consecutive boxes. Samples were stored only by date of line probe assay testing, not by origin or other criteria. We screened records of cultured isolates for those meeting the selection criteria of isoniazid-monoresistant *M tuberculosis* according to MGIT testing, line probe assay, and Xpert MTB/RIF. Among those that met the criteria, we randomly selected 277 frozen isolates for additional testing. Non-consecutive, indiscriminately selected boxes were scanned for isolates belonging to the list of eligible isolates. When a box had been scanned completely, another box was scanned until the total was reached. No random number generation was done (figure 1).

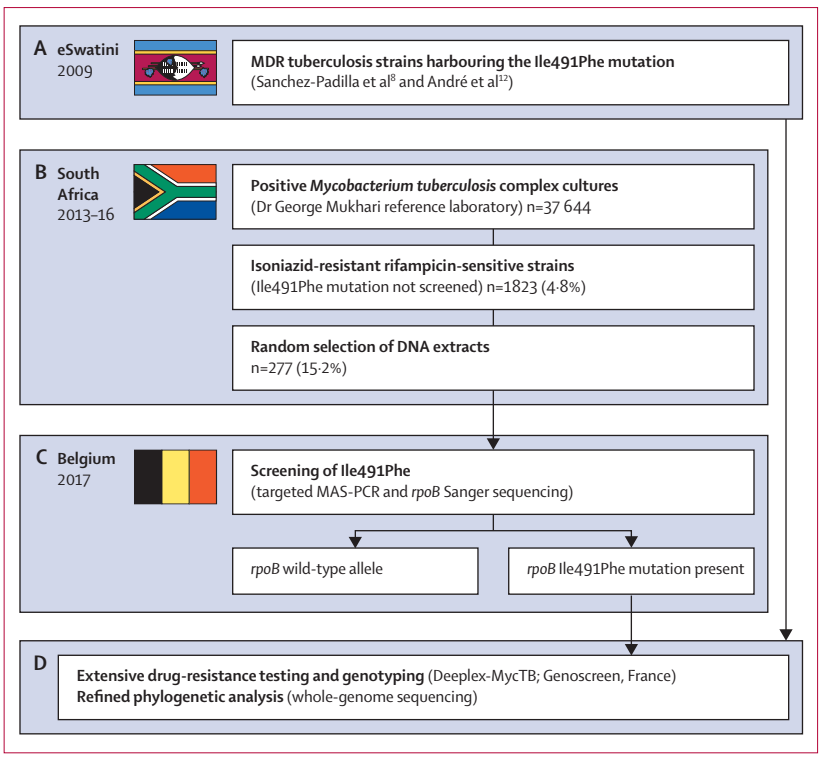

Figure 1: **Selection of samples and flow of tests of strains originating from South Africa and eSwatini**
(A) 17 DNA extracts originating from strains of the 2009 eSwatini survey and previously characterised as MDR tuberculosis associated with the Ile491Phe mutation. (B) 277 DNA extracts of isoniazid-monoresistant strains randomly selected at the Dr George Mukhari reference laboratory. (C) Screening of Ile491Phe *rpoB* mutation with MAS-PCR and Sanger sequencing. (D) Extensive drug-resistance testing and genotyping with the Deeplex-MycTB assay and refined phylogenetic analysis by whole-genome sequencing. MDR=multidrug-resistant. MAS-PCR=multiplex allele-specific PCR.

For DNA extraction, 1 mL of positive BACTEC MGIT 960 culture was centrifuged at 10 000×*g* for 15 min, and the bacterial pellet was resuspended in 100 µL of sterile water and incubated for 20 min at 95°C in a heating block, then in an ultrasonic water bath at room temperature (25°C) for 15 min. After further centrifugation at 10 000×*g* for 15 min the supernatant was transferred into a new tube and stored at –70°C until use.

We also obtained 17 DNA extracts of MDR tuberculosis strains harbouring the Ile491Phe mutation from the 2009 eSwatini study[8] from the Borstel Supranational Reference Laboratory (figure 1).[12]

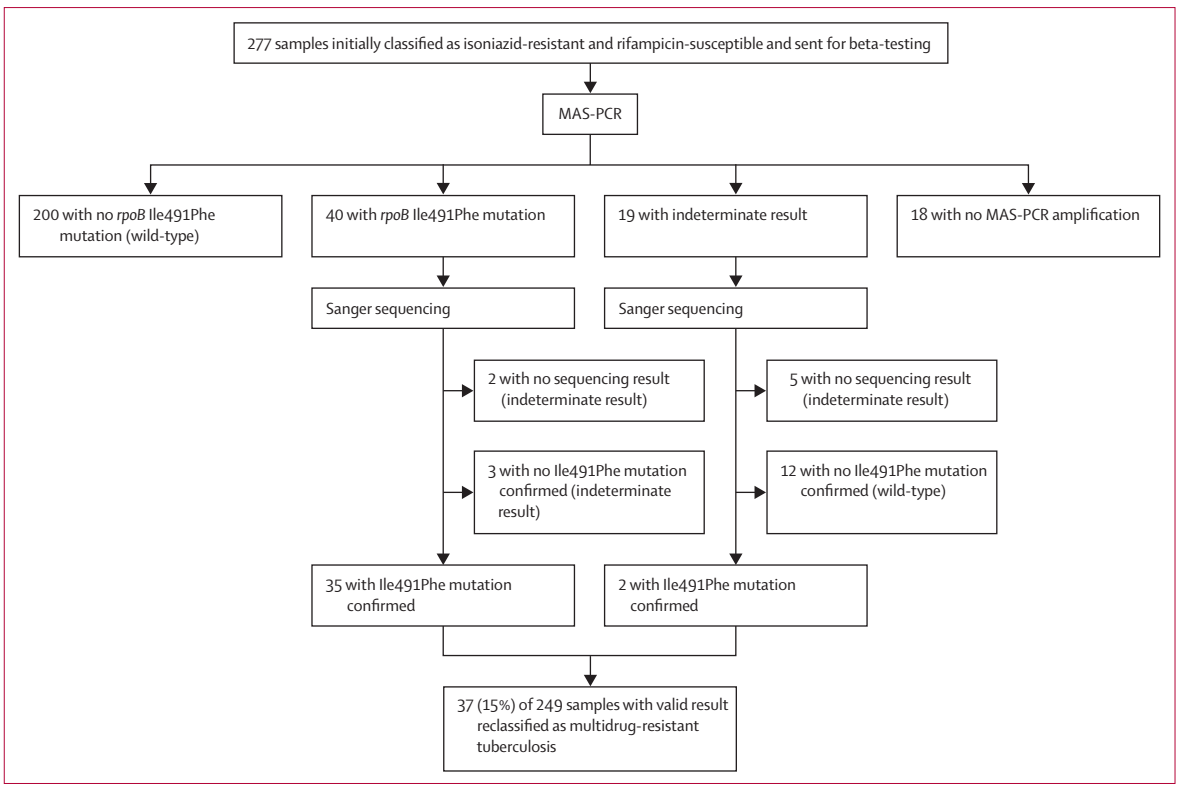

Figure 2: **Results of Ile491Phe screening with a combination of MAS-PCR and Sanger sequencing**
MAS-PCR=multiplex allele-specific PCR.

Multiplex allele-specific PCR and Sanger sequencing

After shipment to the UC Louvain Mycobacteriology Laboratory (Brussels, Belgium), DNA extracts were screened by a rapid multiplex allele-specific (MAS)-PCR technique specifically targeting *rpoB* Ile491Phe.[12] MAS-PCR uses a combination of three primers, resulting in the amplification of allele-specific fragments distinguished by their sizes and melting temperatures. Profiles differing from the defined wild-type or Ile491Phe profiles were considered indeterminate. For all mutant and indeterminate MAS-PCR results, Sanger sequencing of a *rpoB* region including codons 170 and 491 (outside the RRDR) was done (figure 1).

Deeplex-MycTB

Ile491Phe-positive samples from South Africa, identified by Sanger sequencing, and the 17 samples from eSwatini were further analysed by using a beta version of the Deeplex-MycTB kit (Genoscreen, Lille, France) according to manufacturer's instructions (figure 1). This assay uses deep sequencing of a single 24-plexed amplicon mix for simultaneous mycobacterial species identification (based on *hsp65*), genotyping (spoligotyping and phylogenetic single-nucleotide polymorphisms [SNPs]), and prediction of drug resistance of *M tuberculosis* complex strains. 18 gene regions associated with resistance to first-line and second-line drugs are included. Two separate amplicons cover *rpoB* codons 385–516 (encompassing both the RRDR and codon 491) and 158–335. Amplicons were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Indianapolis, IN, USA) and quantified by Qubit dsDNA BR assay (Life Technologies, Paisley, UK). Paired-end libraries of 150-bp read length were prepared with the Nextera XT DNA Sample Preparation kit (Illumina Inc, San Diego, CA, USA) and sequenced on an Illumina MiSeq platform using standard procedures. Variant calling was done with a dedicated parameterised software developed by Genoscreen. Mutations were considered fixed if present in more than 97% of reads, and unfixed (also known as heteroresistance) when present in a lower proportion with a minimum of 3% of all reads.

See Online for appendix

Whole-genome sequencing

Whole-genome sequencing was done on 29 of the 54 Ile491Phe-bearing isolates (14 from South Africa and 15 from eSwatini) that had sufficient amounts and quality of genomic DNA for subsequent genome-wide SNP discovery and refined phylogenetic analysis (appendix).

Statistical analysis

Continuous variables were expressed as mean (SD) and categorical variables as absolute numbers and percentages. Differences in the characteristics of selected and unselected

	Wild-type codon 491 (n=212)	Ile491Phe mutation (n=37)	OR (95% CI)	Adjusted OR* (95% CI)	p value
Sex					
Female	78 (37%)	7 (19%)	Reference	Reference	..
Male	134 (63%)	30 (81%)	2·495 (1·046–5·947]	2·508 (0·915–6·875]	0·07
Province					
Gauteng	83 (39%)	8 (22%)	Reference	Reference	..
Mpumalanga	20 (9%)	2 (5%)	1·037 (0·204–5·267)	..	..
North West	109 (51%)	27 (73%)	2·570 (1·111–5·947)	..	..
Age, years	39·2 (13·4)	35·4 (11·3)	0·978 (0·952–1·005)	0·992 (0·960–1·025)	0·62
HIV status					
Positive	147 (69%)	28 (76%)	Reference	Reference	..
Negative	8 (4%)	7 (19%)	4·594 (1·542–13·689)	9·252 (2·387–35·862)	0·001
Unknown	57 (27%)	2 (5%)	0·184 (0·042–0·799)	0·413 (0·088–1·943)	0·26
Tuberculosis treatment history					
No	159 (75%)	12 (32%)	Reference	Reference	..
Yes	53 (25%)	25 (68%)	6·249 (2·937–13·299)	5·601 (2·356–13·316)	<0·0001
Outcome of current tuberculosis episode					
Combined favourable outcome	64 (30%)	9 (24%)	Reference	Reference	..
Completed treatment	57 (27%)	6 (16%)	..	..	..
Cured	7 (3%)	3 (8%)	..	..	..
Combined unfavourable outcome	36 (17%)	19 (51%)	3·753 (1·538–9·158)	5·363 (1·886–15·256)	0·002
Defaulted	18 (8%)	6 (16%)	..	..	..
Died	9 (4%)	3 (8%)	..	..	..
Failed	9 (4%)	10 (27%)	..	..	..
Combined unknown outcome	112 (53%)	9 (24%)	0·571 (0·216–1·513)	0·910 (0·293–2·828)	0·87
Lost to follow-up	7 (3%)	4 (11%)	..	..	..
Transferred out	15 (7%)	5 (14%)	..	..	..
Unknown	90 (42%)	0 (0%)	..	..	..
Isoniazid resistance					
High or very high	143 (67%)	37 (100%)	NE	NE	NE
Low	69 (33%)	0 (0%)	..	..	..

Data are mean (SD) for age, and number (%) for all categorical variables. OR=odds ratio. NE=non-estimable. *Adjusted from a multivariable model including sex, age, HIV status, tuberculosis treatment history, and outcome of current tuberculosis episode.

Table: **Patient characteristics and factors predictive of *rpoB* Ile491Phe mutation**

subjects were estimated with 95% CIs computed by normal approximation for differences in proportions, and with *t* distribution for differences in means. Logistic regression analysis was used to identify independent predictors of *rpoB* Ile491Phe mutation. The log-linearity assumption was checked for patient age. For HIV status, an unknown category was defined to accommodate missing values in the total set of 249 selected patients. The multivariate model was built by including all predictors and using manual backward selection to reduce the model, maximising 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. Analyses were done with SAS software version 9.4 (SAS Institute Inc, Cary, NC, USA).

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the study data and had final responsibility for the decision to submit for publication.

Results

Of the 37 644 culture-positive samples, 1823 (4·8%) were identified as isoniazid-monoresistant, of which 277 (15·2%) were randomly selected for complementary testing. None of the 1823 isoniazid-monoresistant samples were from Limpopo province.

Selected patients and unselected patients differed by 6·5% (95% CI 0·2 to 12·9) in proportion of male individuals and by –5·3% (–11·7 to 1·2) in proportion of individuals from Gauteng province, and mean age differed by 1·5 years (–0·3 to 3·3; appendix).

For 18 (6%) of 277 samples, no MAS-PCR result could be obtained (figure 2). Of the remaining 259 samples, 200 (77%) had wild-type allele profiles, 40 (15%) showed

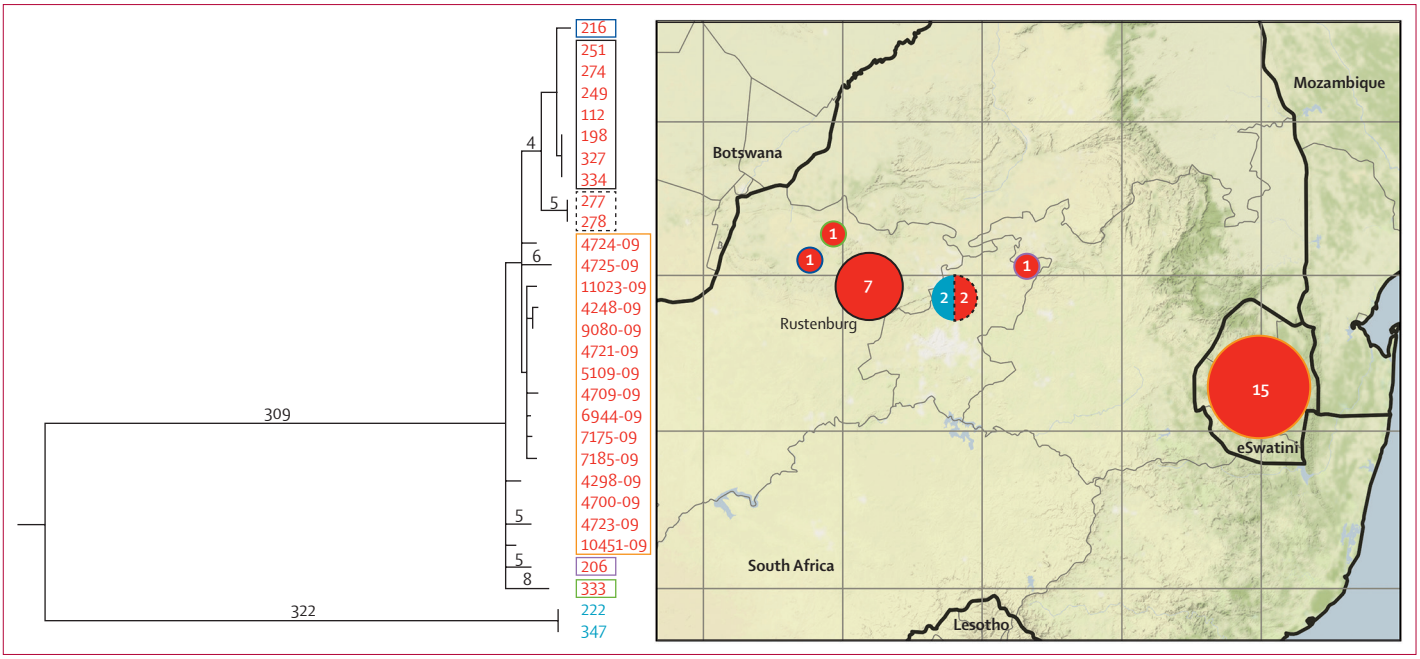

Figure 3: **Genomic distances among *rpoB* Ile491Phe-bearing isolates from South Africa and eSwatini**
Whole-genome sequencing confirmed and further characterised the grouping of strains suggested by the Deeplex-MycTB assay. Isolates of the ST92 group from South Africa (obtained in 2015–16) are shown in blue, and isolates of the ST34 group from South Africa (obtained in 2015–16) and eSwatini (obtained in 2009) are shown in red. Numbers in the neighbour-joining tree indicate single-nucleotide polymorphism distances (when ≥4). The map represents the geographical clustering of strains. For the South African strains, (semi)circles numbered 2 or 7 represent groups within which strains originated from clinics separated by a maximum of 20 km and have identical genomes, or are separated by only one single-nucleotide polymorphism.

a melting temperature shift compatible with Ile491Phe, and 19 (7%) had an indeterminate profile. Sanger sequencing of *rpoB* confirmed the presence of Ile491Phe among 35 (92%) of 38 MAS-PCR-positive and two (14%) of 14 MAS-PCR-indeterminate samples, while no results could be obtained for two (5%) MAS-PCR-positive and five (36%) MAS-PCR-indeterminate samples. On this basis, 37 (15%) of 249 tuberculosis isolates with determinate results and that were previously classified as isoniazid monoresistant by the South African testing algorithm were reclassified as MDR because of the Ile491Phe mutation.

Of the 37 South African isolates, 21 (57%) originated from the Rustenburg municipality in North West province, and the remaining 16 (43%) originated from diverse health facilities in North West, Mpumalanga, and Gauteng provinces. All MDR tuberculosis samples harboured a *katG* Ser315Thr mutation, associated with high-level isoniazid resistance.

Deeplex-MycTB testing was done on the 37 Ile491Phe-bearing South African isolates for concurrent detection of other possible mutations in other drug resistance-associated targets and genotyping. 17 Ile491Phe-bearing isolates from the eSwatini study[13] were also analysed for comparison.

In addition to the concordant detection of Ile491Phe and a common *katG* Ser315Thr mutation conferring isoniazid resistance, all cases had a common *embB* Met306Ile mutation associated with ethambutol resistance and distinct mutations (*pncA* –11A→G or *pncA* His51Asp) conferring pyrazinamide resistance,[14] thus identifying all isolates from South Africa and eSwatini as resistant to all first-line drugs (appendix). *pncA* –11A→G was systematically linked to the *gidB* Leu91Arg mutation and the spoligotype ST92 (part of the so-called X spoligotype clade), and *pncA* His51Asp was systematically associated with *rpsL* Lys43Arg and the spoligotype ST34 (part of the S clade).[15] These observations suggested that the Ile491Phe-bearing MDR tuberculosis strains in South Africa represented two distinct genetic backgrounds. Furthermore, the ST34-matched strain group was disseminated both in eSwatini and across three South African provinces (Gauteng, North West, and Mpumalanga), whereas the ST92-matched group was restricted to South Africa only.

Six of the South African isolates additionally harboured a total of four distinct mutations (Ser2Ile, Cys46Arg, Ala84Ser, and Ala124Glu) in *Rv0678*. These mutations were unfixed in five isolates, detected in proportions of read subpopulations ranging from 3% to 53% within each sample (appendix). By contrast, one Met146Thr mutation was detected among ten isolates from eSwatini. Other variants in *Rv0678* have been associated with decreased sensitivity to bedaquiline.[16]

A history of tuberculosis treatment (adjusted odds ratio 5·601, 95% CI 2·356–13·316, p<0·0001), negative HIV status (9·252, 2·387–35·862, p=0·001), and unfavourable outcome of current tuberculosis episode

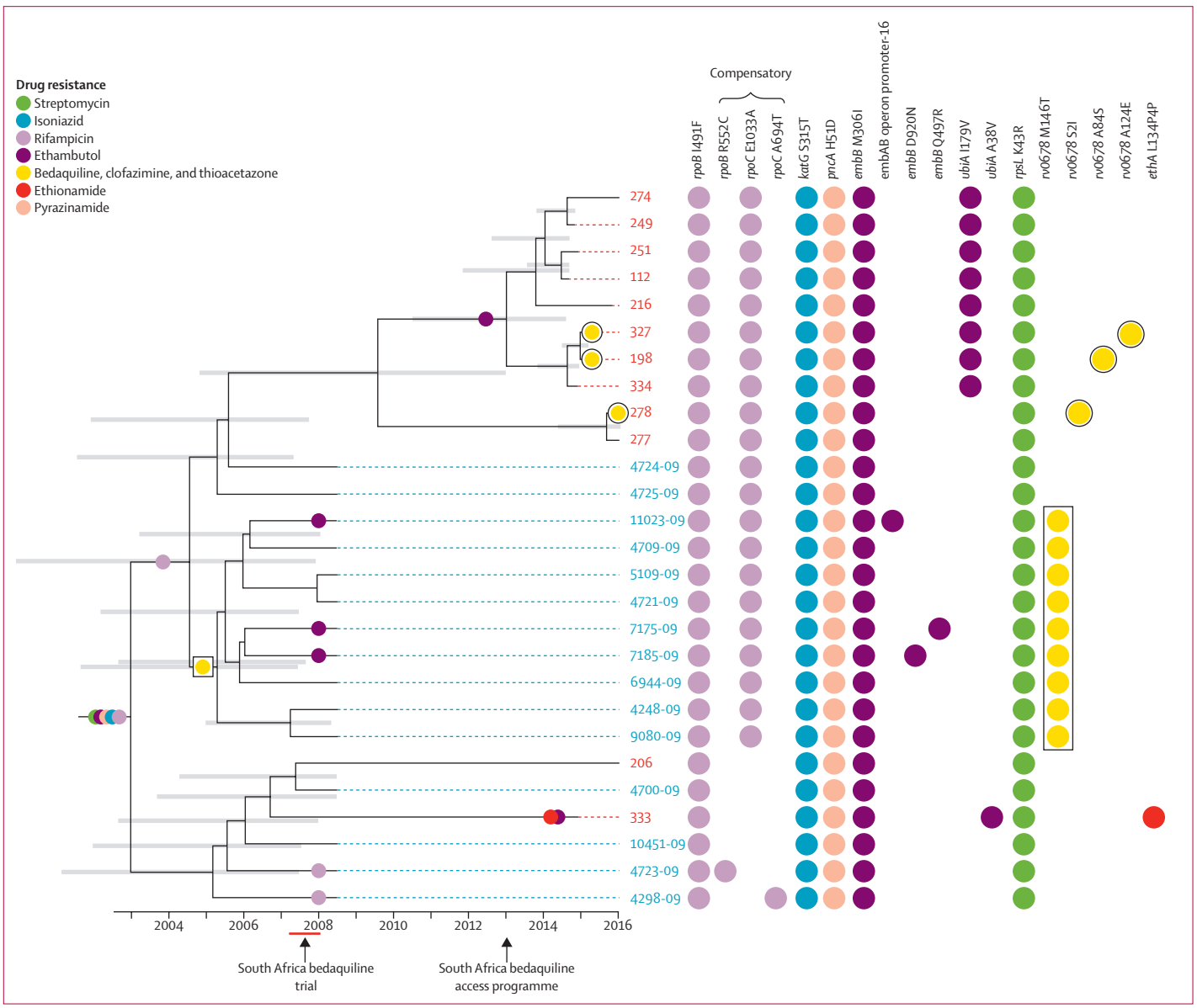

Figure 4: **Dated phylogeny of *rpoB* Ile491Phe-bearing isolates of the ST34 group from South Africa and eSwatini**
The evolutionary history of the isolates was reconstructed with a Bayesian tree with a corresponding time scale shown at the bottom. Isolates from South Africa are shown in red and those from eSwatini are shown in blue. Dotted lines show the correspondence between isolates and terminal parts of tree branches. Grey bars indicate the 95% highest posterior density intervals on nodal positions. Events of drug-resistance mutation acquisition (coloured circles) were positioned on the tree based on parsimony. The accumulation of drug-resistant mutations within a strain is denoted to the right of the tree. Mutations acquired in the *Rv0678* gene (associated with resistance to bedaquiline, clofazimine, and thioacetazone), or putative compensatory mutations, are shown inside black circles (independent emergences in individual isolates from South Africa) or a black box (single emergence transmitted vertically in eSwatini). Dates of the phase 2 clinical trial and of the start of the clinical access programme for bedaquiline treatment of patients with drug-resistant tuberculosis in South Africa are indicated by arrows below the time scale.

(5·363, 1·886–15·256, p=0·002) were independent predictive factors of the Ile491Phe mutation after adjustment for age and sex (table).

Exploitable whole-genome sequencing data could be obtained from 29 (54%) of 54 isolates, including 15 (88%) of 17 from eSwatini and 14 (38%) of 37 from South Africa. Upon reconstruction of genomic distances, a minimum of 631 SNPs were found to separate the analysed ST34 and ST92 isolates (figure 3), showing that Ile491Phe arose independently in two lineages, identified as 4.4.1.1 and 4.1.1.3 (part of the Euro-American branch of the *M tuberculosis* complex) on the basis of reference SNP barcodes.[17] This identification is consistent with the known correspondence of the 4.4 lineage to the S spoligotype clade, and the 4.1.1 lineage to the X spoligotype clade.[18]

By contrast, the analysed S clade ST34 isolates from South Africa and eSwatini formed a single compact

genomic cluster in which no more than six SNPs separated a patient isolate from that of at least one other patient in the cluster (figure 3). According to reference genomic studies,[18,19] such restricted variation indicates that these isolates are all part of a same transmission network. Of note, basal positions in the reconstructed trees were occupied by isolates from eSwatini collected in 2009, suggesting that this region represents the historical and geographical origin of the outbreak. However, different subsets of South African isolates obtained 6–7 years later had identical genomes or were separated only by a single SNP. Isolates within these subsets were found to be geographically clustered within 20 km (figure 3), suggesting later, onward spread in South Africa.

We further estimated the chronology of this evolutionary scenario for this cluster by applying a Bayesian statistical approach (figure 4). Using the available isolation dates, we calculated that ST34 mutated at a rate of 0·85 SNPs per genome per year (95% highest posterior density interval 0·38–1·36), within the 0·3–1·3 range reported in the literature.[19–22] Applying this rate, we estimated that the most recent common ancestor of the analysed ST34 strains emerged in eSwatini around 2002, and South African isolates diverged from those from eSwatini between roughly 2006 and 2009, with multiple transmission events in South Africa between 2013 and 2016.

Three distinct *Rv0678* mutations in the South African isolates subjected to whole-genome sequencing were mapped at tips of the reconstructed trees, with acquisition estimated to be after programmatic access to bedaquiline in March, 2013. By contrast, the single *Rv0678* mutation (Met146Thr) present in nine analysed isolates from eSwatini resulted from a single emergence predating the start of the bedaquiline programme. Finally, we detected at least three distinct acquisition events of mutations in *rpoA*, *rpoB* (outside the RRDR), or *rpoC* along the evolution of ST34 strains from both settings, putatively compensating for the fitness cost of rifampicin resistance,[23,24] in this case due to Ile491Phe.

Discussion

In the context of Xpert MTB/RIF roll-out, WHO has recommended use of rifampicin resistance as a surrogate marker for MDR tuberculosis. This study reports the emergence and spread in South Africa of MDR tuberculosis strains with a particular mutation, Ile491Phe, that is associated with rifampicin resistance but missed by the Xpert MTB/RIF and all other commercial molecular and phenotypic assays currently endorsed by WHO. Our whole-genome sequencing data show that this mutation arose independently in two distinct lineages in South Africa, only one of which represents the direct extension of the outbreak detected in eSwatini.[13]

Importantly, this previously known mutation associated with rifampicin resistance has not been taken into account when validating Xpert MTB/RIF.[25] Moreover, surveillance programmes have so far failed to monitor its emergence, as is the case in South Africa, where surveillance based on whole-genome sequencing is done only on cultured strains classified as rifampicin resistant by Xpert MTB/RIF testing. Alarmingly, the 2018 WHO guidelines for the treatment of isoniazid-resistant tuberculosis suggest a 6-month first-line regimen of rifampicin, ethambutol, and pyrazinamide throughout, strengthened by levofloxacin.[26] In occult MDR tuberculosis caused by Ile491Phe-harbouring strains resistant to all first-line drugs, as in this study, the aforementioned regimen would be equivalent to fluoroquinolone (levofloxacin) monotherapy, rapidly resulting in resistance to this core drug of MDR tuberculosis treatment regimens (ie, pre-extensively drug-resistant tuberculosis).[27]

In all tested isolates from South Africa and eSwatini, we found that this mutation was systematically associated with a high-level isoniazid-resistance mutation, as well as with mutations conferring resistance to the two other first-line anti-tuberculosis drugs pyrazinamide and ethambutol. Resistance to all first-line drugs was unreported in the original study on eSwatini strains. This systematic association strongly suggests that amplification of resistance resulted from a repeatedly ineffective first-line treatment regimen, following failed diagnosis of the rifampicin-resistance genotype.

As a predictable consequence, 68% of patients infected by these strains in this study had undergone at least one unsuccessful previous first-line tuberculosis treatment. These findings are concordant with previous studies reporting poor outcomes among patients carrying strains with Ile491Phe and other *rpoB* mutations missed by phenotypic testing.[28] Patients with prolonged periods of inadequate treatment expectedly contribute to the active spread of undiagnosed MDR tuberculosis strains in the community. This conclusion is supported by our results of whole-genome sequencing analysis, indicating onward transmission of such strains in the studied South African settings. Moreover, our genomic data show that all analysed ST34 isolates from South Africa and eSwatini are part of the same longitudinal outbreak, probably originating from eSwatini and dating back to at least 2002, thus indicating spread of these strains from eSwatini to neighbouring South African provinces over more than a decade.

Deeplex-MycTB deep sequencing revealed that half the mutations among the ST34 genotypic variants from South Africa or eSwatini were distinct missense mutations in *Rv0678*, either fixed or (as yet) unfixed within individual samples. Our phylogenetic reconstruction based on whole-genome sequencing showed that at least three such mutations each arose as the sole detectable SNP in groups of otherwise indistinguishable genomes from South Africa, at estimated dates after the programmatic introduction of bedaquiline for the treatment of selected patients with drug-resistant tuberculosis in March, 2013. Although the association of these mutations with drug resistance is

unknown, these data indicate ongoing positive selection acting on *Rv0678* in this strain population, linked to the introduction of bedaquiline. We also detected an earlier, single emergence followed by vertical transmission of another *Rv0678* mutation in a ST34 subgroup from eSwatini, possibly reflecting sporadic use of, and acquired cross-resistance to, thioacetazone or clofazimine,[29,30] which are not recommended by local national programmes for routine use in drug-resistant tuberculosis treatment.

One limitation of this study was that, although it monitored the presence of the Ile491Phe mutation in some South African provinces, it was not designed to elucidate the exact prevalence of this mutation and the burden of undetected MDR tuberculosis in the whole country. Further studies are warranted to assess the prevalence of this and other mutations external to the RRDR that confer rifampicin resistance. The latest national drug resistance survey in South Africa,[11] which was not designed to detect Ile491Phe-related rifampicin resistance, identified 17 970 isoniazid-monoresistant cases. Given the results presented in our study, a substantial number of samples currently classified as isoniazid-monoresitant in South Africa might in fact be MDR tuberculosis. However, more data are needed to elucidate whether the high proportion of these undetected MDR tuberculosis cases found in the present study are contained in particular geographical areas or not.

Data retrieved from a whole-genome sequencing study of 5310 isolates from five continents, published in 2017, indicated a total frequency of 2·8% for resistance-associated mutations at codons 491, 493, and 170—all missed by current commercial molecular tests—among rifampicin-resistant strains.[5] In this dataset, 20 independent emergence events of strains bearing one of these mutations could be counted at least in Africa, Asia, and Europe. These figures might, nevertheless, be underestimates of the true prevalence, as many regions were under-sampled or not represented and a phenotypic preselection was presumably done for interesting resistance profiles (selecting against isolates for which rifampicin resistance was not detected using programmatic algorithms). However, even if the prevalence of these mutations were to be generally low at present, strains harbouring these mutations are predicted to be selected at population level as a result of their escape from current diagnostics being deployed globally, unless implementation strategies are improved.

Mutations missed by MGIT-based rifampicin-susceptibility testing are presumed to have a greater fitness cost than the common mutations (eg, in *rpoB* codon 450), even though they are associated with adverse treatment outcomes.[24] However, the effect of such fitness cost is expected to be diminished in settings, such as South Africa, that have a high HIV infection prevalence and, thus, many weakened hosts. Moreover, the observed acquisition of mutations in *rpoA*, *rpoB* (outside the RRDR), or *rpoC* during evolution of ST34 isolates over the past decade suggests that the cost of Ile491Phe could now be mitigated in some strains, thereby increasing their transmissibility. Therefore, the epidemiological and genetic contexts and the current phenotypic and molecular algorithms together could provide a so-called perfect storm for such selective diagnostic pressure to lead to more escaped clones, as we have shown here.

Our findings highlight a serious gap in current WHO-endorsed algorithms for capturing all MDR tuberculosis strains. This gap results in inappropriate tuberculosis treatment, amplification of drug resistance, and adverse treatment outcomes. Our observations highlight the importance for South Africa and other countries to monitor the prevalence and potential increase in frequency of tuberculosis strains bearing rifampicin-resistance mutations outside the RRDR in drug-resistance surveys, without phenotypic preselection for rifampicin resistance. The obtained data will better guide and complement the implementation of available tools. Newer tools such as whole-genome sequencing or the Deeplex-MycTB multitarget assay should be used in such surveys to help to close existing gaps.

Contributors

All authors are collaborators and part of the study and contributed to the writing of the Article. NAM, EM, PS, BCdJ, and EA designed the study. The supervision of the strain collection, initial testing, and selection were done by NAM, EM, MN, and LR (South Africa) and GM, PB, and SN (eSwatini). FB, LG, CG, PS, and EA did the confirmatory testing. Data collection was done by NAM, EM, LR, EA, PS, FB, GM, PB, SN, and LG. NAM, EM, MF, J-CD, CP, LR, EA, BCdJ, CG, AJ, PS, FB, MD, and LG managed and analysed the data. All authors participated in writing the Article. PS, BCdJ, and EA jointly supervised the manuscript.

Declaration of interests

PS is a consultant for Genoscreen. CG and AJ are employees of Genoscreen. All other authors declare no competing interests.

Acknowledgments

This study was funded by VLIR-UOS (to NAM), National Research Foundation (South Africa; to NAM [81974]), and Innoviris (to EA, J-CD, MF, FB, and MD). BCdJ was supported by a European Research Council starting grant for INTERRUPTB (311725), and Genoscreen was supported by the French governmental programme Investing for the Future. EA was an employee of Cliniques Universitaires Saint-Luc (UC Louvain, Belgium) at the start of this study. We thank all colleagues at the Department of Medical Microbiology, National Health Laboratory Service–Dr George Mukhari Tertiary Laboratory; the Department of Microbiological Pathology, Sefako Makgatho Health Sciences University; the Mycobacteriology Unit, Institute of Tropical Medicine (Antwerp, Belgium); and the Global Health Institute, University of Antwerp. We thank all patients who were part of this study and the respective clinics and hospitals in Gauteng, North West, and Mpumalanga provinces; Elisabeth Sanchez-Padilla and Maryline Bonnet (Epicentre, Médecins Sans Frontières, Paris); and Rosine Gros (UC Louvain, Belgium, and Université Claude Bernard Lyon 1, France) and Stéphanie Duthoy (Genoscreen, Lille, France) for their technical assistance.

References

1 Chakravorty S, Simmons AM, Rowneki M, et al. The new Xpert MTB/RIF Ultra: improving detection of *Mycobacterium tuberculosis* and resistance to rifampin in an assay suitable for point-of-care testing. *MBio* 2017; **8:** e00812–17.

2 Nathavitharana RR, Hillemann D, Schumacher SG, et al. Multicenter noninferiority evaluation of Hain GenoType MTBDRplus Version 2 and Nipro NTM+ MDRTB line probe assays for detection of rifampin and isoniazid resistance. *J Clin Microbiol* 2016; **54:** 1624–30.

3 Telenti A, Imboden P, Marchesi F, et al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 1993; **341:** 647–50.

4 Andre E, Goeminne L, Cabibbe AM, et al. Consensus numbering system for the rifampicin resistance-associated rpoB gene mutations in pathogenic mycobacteria. *Clin Microbiol Infect* 2017; **23:** 167–72.

5 Manson AL, Cohen KA, Abeel T, et al. Genomic analysis of globally diverse *Mycobacterium tuberculosis* strains provides insights into the emergence and spread of multidrug resistance. *Nat Genet* 2017; **49:** 395–402.

6 Andre E, Martin A. Isoniazid-resistant tuberculosis treatment with first-line drugs. *Lancet Infect Dis* 2017; **17:** 258–59.

7 Feliciano CS, Namburete EI, Plaça JR, et al. Accuracy of whole genome sequencing versus phenotypic (MGIT) and commercial molecular tests for detection of drug-resistant *Mycobacterium tuberculosis* isolated from patients in Brazil and Mozambique. *Tuberculosis (Edinb)* 2018; **110:** 59–67.

8 Sanchez-Padilla E, Merker M, Beckert P, et al. Detection of drug-resistant tuberculosis by Xpert MTB/RIF in Swaziland. *N Engl J Med* 2015; **372:** 1181–82.

9 Rigouts L, Gumusboga M, de Rijk WB, et al. Rifampin resistance missed in automated liquid culture system for *Mycobacterium tuberculosis* isolates with specific rpoB mutations. *J Clin Microbiol* 2013; **51:** 2641–45.

10 Van Deun A, Aung K, Hossain A, et al. Disputed rpoB mutations can frequently cause important rifampicin resistance among new tuberculosis patients. *Int J Tuberc Lung Dis* 2015; **19:** 185–90.

11 Ismail NA, Mvusi L, Nanoo A, et al. Prevalence of drug-resistant tuberculosis and imputed burden in South Africa: a national and sub-national cross-sectional survey. *Lancet Infect Dis* 2018; **18:** 779–87.

12 André E, Goeminne L, Colmant A, Beckert P, Niemann S, Delmee M. Novel rapid PCR for the detection of Ile491Phe rpoB mutation of *Mycobacterium tuberculosis*, a rifampicin resistance-conferring mutation undetected by commercial assays. *Clin Microbiol Infect* 2017; **23:** 267.e5–e7.

13 Sanchez-Padilla E, Dlamini T, Ascorra A, et al. High prevalence of multidrug-resistant tuberculosis, Swaziland, 2009–2010. *Emerg Infect Dis* 2012; **18:** 29–37.

14 Yadon AN, Maharaj K, Adamson JH, et al. A comprehensive characterization of PncA polymorphisms that confer resistance to pyrazinamide. *Nat Commun* 2017; **8:** 588.

15 Demay C, Liens B, Burguière T, et al. SITVITWEB—a publicly available international multimarker database for studying *Mycobacterium tuberculosis* genetic diversity and molecular epidemiology. *Infect Genet Evol* 2012; **12:** 755–66.

16 Villellas C, Coeck N, Meehan CJ, Lounis N, de Jong B, Rigouts L, et al. Unexpected high prevalence of resistance-associated Rv0678 variants in MDR-TB patients without documented prior use of clofazimine or bedaquiline. *J Antimicrob Chemother* 2017; **72:** 684–90.

17 Coll F, McNerney R, Guerra-Assuncao JA, et al. A robust SNP barcode for typing *Mycobacterium tuberculosis* complex strains. *Nat Commun* 2014; **5:** 4812.

18 Niemann S, Merker M, Kohl T, Supply P. Impact of genetic diversity on the biology of *Mycobacterium tuberculosis* complex strains. *Microbiol Spectr* 2016; **4:** TBTB2-0022-2016.

19 Roetzer A, Diel R, Kohl TA, et al. Whole genome sequencing versus traditional genotyping for investigation of a *Mycobacterium tuberculosis* outbreak: a longitudinal molecular epidemiological study. *PLoS Med* 2013; **10:** e1001387.

20 Walker TM, Ip CL, Harrell RH, et al. Whole-genome sequencing to delineate *Mycobacterium tuberculosis* outbreaks: a retrospective observational study. *Lancet Infect Dis* 2013; **13:** 137–46.

21 Eldholm V, Monteserin J, Rieux A, et al. Four decades of transmission of a multidrug-resistant *Mycobacterium tuberculosis* outbreak strain. *Nat Commun* 2015; **6:** 7119.

22 Eldholm V, Pettersson JH-O, Brynildsrud OB, et al. Armed conflict and population displacement as drivers of the evolution and dispersal of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 2016; **113:** 13881–86.

23 Coll F, Phelan J, Hill-Cawthorne GA, et al. Genome-wide analysis of multi-and extensively drug-resistant *Mycobacterium tuberculosis*. *Nat Genet* 2018; **50:** 307–16.

24 Comas I, Borrell S, Roetzer A, et al. Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. *Nat Genet* 2011; **44:** 106–10.

25 Boehme CC, Nabeta P, Hillemann D, et al. Rapid molecular detection of tuberculosis and rifampin resistance. *N Engl J Med* 2010; **363:** 1005–15.

26 WHO. WHO treatment guidelines for isoniazid-resistant tuberculosis: supplement to the WHO treatment guidelines for drug-resistant tuberculosis. Geneva: World Health Organization, 2018.

27 Van Deun A, Decroo T, Piubello A, de Jong B, Lynen L, Rieder H. Principles for constructing a tuberculosis treatment regimen: the role and definition of core and companion drugs. *Int J Tuberc Lung Dis* 2018; **22:** 239–45.

28 Van Deun A, Aung KJ, Bola V, et al. Rifampin drug resistance tests for tuberculosis: challenging the gold standard. *J Clin Microbiol* 2013; **51:** 2633–40.

29 Hartkoorn RC, Uplekar S, Cole ST. Cross-resistance between clofazimine and bedaquiline through upregulation of MmpL5 in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2014; **58:** 2979–81.

30 Halloum I, Viljoen A, Khanna V, et al. Resistance to thiacetazone derivatives active against *Mycobacterium abscessus* involves mutations in the MmpL5 transcriptional repressor MAB_4384. *Antimicrob Agents Chemother* 2017; **61:** e02509–16.