Emergence of bedaquiline-resistant tuberculosis and of multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis* strains with *rpoB* Ile491Phe mutation not detected by Xpert MTB/RIF in Mozambique: a retrospective observational study

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Summary

Background In 2021, an estimated 4800 people developed rifampicin-resistant tuberculosis in Mozambique, 75% of which went undiagnosed. Detailed molecular data on rifampicin-resistant and multidrug-resistant (MDR) tuberculosis are not available. Here, we aimed at gaining precise data on the determinants of rifampicin-resistant and MDR tuberculosis in Mozambique.

Methods In this retrospective observational study, we performed whole-genome sequencing of 704 rifampicin-resistant *Mycobacterium tuberculosis* complex (Mtbc) strains submitted to the National Tuberculosis Reference Laboratory (NTRL) in Maputo, Mozambique, between 2015 and 2021. Phylogenetic strain classification, genomic resistance prediction, and cluster analysis were performed.

Findings Between Jan 1, 2015, and July 31, 2021, 2606 Mtbc isolates with an isoniazid or rifampicin resistance were identified in the NTRL biobank, of which, 1483 (56·9%) were from men, 1114 (42·7%) from women, and nine (0·4%) were unknown. Genome-based drug-resistant prediction classified 704 Mtbc strains as rifampicin resistant. 628 (89%) of the 704 Mtbc strains were classified MDR; of those, 146 (23%) were pre-extensively drug resistant (pre-XDR; additional fluoroquinolone resistance), and 24 (4%) extensively drug resistant (XDR; combined fluoroquinolone and bedaquiline resistance). Overall, 61 (9%) of 704 strains revealed resistance to bedaquiline: five (7%) of 76 rifampicin resistant plus bedaquiline resistant, 32 (7%) of 458 MDR plus bedaquiline resistant, and 24 (100%) of 24 XDR. Prevalence of bedaquiline resistance increased from 3% in 2016 to 14% in 2021. The cluster rate (12 single-nucleotide polymorphism threshold) was 42% for rifampicin-resistant strains, 78% for MDR strains, 94% for pre-XDR strains, and 96% for XDR Mtbc strains. 31 (4%) of 704 Mtbc strains, belonging to a diagnostic escape outbreak strain previously described in Eswatini (group_56), had an *rpoB* Ile491Phe mutation which is not detected by Xpert MTB/RIF (no other *rpoB* mutation). Of these, 23 (74%) showed additional resistance to bedaquiline, 13 (42%) had bedaquiline and fluoroquinolone resistance, and two (6%) were bedaquiline, fluoroquinolone, and delamanid resistant.

Interpretation Pre-XDR resistance is highly prevalent among MDR Mtbc strains in Mozambique and so is bedaquiline resistance; and the frequency of bedaquiline resistance quadrupled over time and was found even in Mtbc strains without fluoroquinolone resistance. Importantly, strains with Ile491Phe mutation were frequent, accounting for 31% (n=10) of MDR plus bedaquiline-resistant strains and 54% (n=13) of XDR Mtbc strains. Given the current diagnostic algorithms and treatment regimens, both the emergence of rifampicin resistance due to Ile491Phe and bedaquiline resistance might jeopardise MDR tuberculosis prevention and care unless sequencing-based technology is rolled out. The potential cross border spread of diagnostic escape strains needs further investigation.

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Introduction

COVID-19 had briefly overtaken tuberculosis as the leading infectious disease killer in 2020; however, tuberculosis has regained its lead, despite being both preventable and curable, especially if susceptible to all first-line drugs.[1] For the first time in a decade, the

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Research in context

Evidence before this study

We searched PubMed for articles using the terms "Mozambique", and "MDR TB", from database inception up to May 26, 2023. No language restrictions were applied to this search. Mozambique is particularly affected by the tuberculosis epidemic, with one of the highest tuberculosis incidences (368 cases per 100 000 population) in the WHO African region. Consequently, there are substantial challenges to diagnose and treat people with tuberculosis. The problem is even worse considering the large numbers of people with active multidrug-resistant (MDR) tuberculosis. In Mozambique, an estimated 4800 people have new rifampicin-resistant or MDR tuberculosis every year, of which only a quarter of infections are laboratory confirmed, indicating a substantial gap in the case detection system. This gap could lead to a substantial increase of drug-resistant tuberculosis. Also, new drug-resistant tuberculosis treatment regimens such as the MDR treatment endorsed by WHO (Dec 15, 2022) including novel antibiotics (eg, bedaquiline and linezolid) might become ineffective due to resistance development over comparably short time frames which would have a profound effect on individuals with either MDR or rifampicin-resistant tuberculosis. Such a scenario is alarming, in that it will threaten the care and treatment of people affected by drug-resistant tuberculosis and facilitate transmission of drug-resistant tuberculosis in Mozambique and the WHO African region. To prevent its occurrence, detailed data on the MDR tuberculosis epidemiology are needed to better guide interventions to limit the spread of MDR tuberculosis. However, longitudinal studies applying whole-genome sequencing (WGS) for molecular epidemiology of rifampicin resistant or MDR strains have not yet been performed in Mozambique.

Added value of this study

To understand the determinants of the MDR tuberculosis epidemic, we performed WGS of 704 rifampicin-resistant *Mycobacterium tuberculosis* complex (Mtbc) strains, 628 of which were classified as MDR, submitted to the National Tuberculosis Reference Laboratory in Maputo, Mozambique, between 2015 and 2021. We found a high rate of pre-extensively drug-resistant (pre-XDR; 146 [23%]) strains among the 628 MDR strains, and that bedaquiline resistance has increased from 3% in 2016 to 14% in 2021. Further, we detected 42 Mtbc strains with a diagnostic escape *rpoB* Ile491Phe mutation, that is not detected by Xpert Mtb/RIF. Ile491Phe strains already represent 31% of the MDR plus bedaquiline-resistant strains, and 54% of the extensively drug-resistant (XDR) strains in the study population. Additionally, we found that cluster rates were high with 42% rifampicin-resistant, 78% MDR, 94% pre-XDR, and 96% XDR Mtbc strains in clusters, suggesting recent transmission as the main driver of the drug-resistant tuberculosis epidemic in the country. Transmission of all strain types (MDR, pre-XDR, and XDR) could be further confirmed by identical mutation patterns.

Implications of all the available evidence

Our data document that pre-XDR is highly prevalent among MDR Mtbc strains in Mozambique and so is bedaquiline resistance, which quadrupled in frequency over time and was found even in Mtbc strains without fluoroquinolone resistance. Such high levels of resistance to fluoroquinolone and bedaquiline, in combination with resistance to all first line antibiotics (isoniazid, rifampicin, ethambutol, and pyrazinamide), are likely to threaten the national roll-out of the new WHO approved 6 months regimens of bedaquiline, pretomanid, linezolid, and moxifloxacin or bedaquiline, pretomanid, and linezolid. Importantly, bedaquiline resistance in Mozambique was not limited to fluoroquinolone-resistant strains, suggesting that current treatment regimens are unable to prevent development of resistance on a population level, which has serious implications for current MDR tuberculosis therapy on a global level. An equally worrisome finding is the high proportion of Mtbc strains with the Ile491Phe mutation among MDR plus bedaquiline-resistant and XDR Mtbc strains, which remains undetected by Xpert MTB/RIF and commercially available line probe assays because most of them have no other canonical *rpoB* mutation. Given the current diagnostic algorithms and treatment regimens, both the emergence of rifampicin resistant due to Ile491Phe and bedaquiline resistance in general might jeopardise efforts to contain the MDR-tuberculosis epidemic in Mozambique. Comparison with strains from South Africa and Eswatini supports cross border spread, a finding that needs to be further investigated.

number of people with tuberculosis and tuberculosis associated deaths have risen as a result of reduced access to and provision of essential tuberculosis services, including diagnostics, during the COVID-19 pandemic.[1]

The global tuberculosis pandemic is worsened by the emergence and spread of drug-resistant, multidrug-resistant (MDR; resistance to at least isoniazid and rifampicin), pre-extensively drug-resistant (pre-XDR; MDR plus resistance to any fluoroquinolone), and extensively drug-resistant (XDR; pre-XDR plus additional resistance to at least one of additional Group A drugs bedaquiline or linezolid) tuberculosis.[1]

MDR tuberculosis programmes are challenged by diagnostic delays, limited availability of reliable drug susceptibility testing for drugs included in standardised treatment regimens, underdosing due to fixed or weight-band dosing and a scarcity of drug monitoring, long duration of and poor adherence to treatment, frequent adverse events, and high morbidity and mortality.[2] Delay in diagnosing drug resistance together with non-effective or suboptimal treatment amplifies resistances

and results in treatment failure and MDR tuberculosis transmission.[2]

In May, 2022, WHO recommended a novel all-oral 6-month regimen of bedaquiline, pretomanid, and linezolid plus moxifloxacin (in the absence of fluoroquinolone resistance) for treatment of MDR tuberculosis.[3] This new regimen was found to be non-inferior to standard 9 months and 12 months MDR tuberculosis regimens and, in some trials, was found to be favourable because of a reduction in adverse events. Although this new regimen holds great promise, the emergence of fluoroquinolone and bedaquiline resistance described in 2022 might threaten the longevity of the regimen.[4,5]

Thus, understanding the underlying prevalence of fluoroquinolone and bedaquiline resistance before introducing the new regimen is crucial.[6,7] Equally important is the ongoing monitoring of resistance development—eg, fostered by the transmission of *Mycobacterium tuberculosis* complex (Mtbc) strains with particular resistance profiles that can negatively impact diagnostic or treatment strategies in a given geographical region, as shown for the Ile491Phe mutation strain in Eswatini.[8] The Ile491Phe mutation is not detected by conventional rapid molecular drug susceptibility tests or by the Xpert MTB/RIF assay.[8]

With an estimated tuberculosis incidence of 368 per 100 000 population, an HIV prevalence of 12·4%,[9] and an estimated 4800 people who newly develop MDR tuberculosis each year, Mozambique, a country in sub-Saharan Africa, belongs to the 30 high tuberculosis, HIV tuberculosis, and MDR tuberculosis burden countries.[1] One in four people with MDR tuberculosis in Mozambique remained undiagnosed in 2021.[10] Despite the severe implications of drug resistance, detailed information on drug resistance determinants is not available.

To address the determinants of drug resistance, we aimed to do a retrospective genomic epidemiological analysis based on whole-genome sequencing (WGS) of 809 drug-resistant Mtbc strains submitted to the National Tuberculosis Reference Laboratory (NTRL) in Maputo, Mozambique, for drug susceptibility testing between 2015 and 2021. WGS data were used to determine Mtbc lineage, resistance profiles and mechanisms, and transmission inference based on genome-based cluster analysis.

Methods

Study design and population

In this retrospective observational study, Mtbc isolates from distinct people with tuberculosis, submitted to the NTRL in Maputo, Mozambique, between Jan 1, 2015, and July 31, 2021, with at least isoniazid or rifampicin resistance, or both, determined by Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA), line probe assay Genotype MDRplus (Hain Lifescience [a Bruker company], Nehren, Germany), or phenotypic drug susceptibility testing were eligible for inclusion. Sex was self-reported by the participants (male or female).

Mozambique has three regional tuberculosis reference laboratories: the NTRL in Maputo, one in the central region (Beira), and one in the northern region (Nampula). The NTRL primarily receives samples from Maputo and Gaza. The national algorithm recommends sending samples for culture and drug susceptibly testing of all people with evidence of rifampicin-resistant tuberculosis (as per Xpert MTB/RIF testing), people with positive smear results at 2 months of treatment, and people with disease relapse and treatment failure.

All Mtbc strains isolated at the NTRL are routinely stored in glycerol at –80°C. Mtbc strains were identified through the laboratory information system, subcultured on Löwenstein-Jensen medium, and if culture was successful DNA was extracted using the cetyltrimethylammonium bromide-lysozyme method.[11]

No patient information was included in this analysis, and data was anonymised at the source. The study protocol and ethics were approved by the National Bioethics Committee for Health in Maputo, Mozambique (260/CNBS/20).

WGS

WGS was performed on NextSeq 500/2000 (Illumina, San Diego, CA, USA) machines using a modified Nextera-based library preparation workflow.[12] WGS data were analysed using the MTBseq pipeline as described previously.[13]

Genome-based resistance prediction and cluster analysis

Data processing for variant calling and inference of transmission events was done as previously described.[14] For genome-based resistance, prediction polymorphisms in 27 drug resistance associated genes that are involved in drug resistance mechanisms were determined from the WGS data using a previously published interpretation catalogue (appendix 1).[4] After applying the expert rules for bedaquiline, unknown mutations in genes associated with resistance such as *atpE* or *Rv0678* were reclassified using an extended bedaquiline resistance catalogue. Further details of these methods are in appendix 2 (p 2).[15]

See Online for appendix 1

See Online for appendix 2

Phylogenetic inference

Phylogenetic Mtbc lineages and sublineages were inferred from signature single-nucleotide polymorphisms (SNPs).[16,17] Strains were grouped in SNP clusters by considering a maximum pairwise genetic distance between at least two Mtbc isolates of five or fewer SNPs (d5 cluster) and 12 or fewer SNPs (d12 cluster) as indicator for tuberculosis infections associated with direct transmission events.[18,19] Phylogenetic trees were calculated based on concatenated SNP alignments. For the coalescent-based analyses and the inference of the

	Total	Rifampicin resistant	Rifampicin resistant plus bedaquiline resistant	Multidrug resistant	Multidrug resistant plus bedaquiline resistant	Pre-extensively drug resistant	Extensively drug resistant
Dataset*	704 (100%)	71 (10·1%)	5 (0·7%)	426 (60·5%)	32 (4·5%)	146 (20·7%)	24 (3·4%)
Lineage†							
Lineage 1	66 (9·4%)	12 (16·9%)	0	48 (11·3%)	2 (6·3%)	4 (2·7%)	0
Lineage 2	154 (21·9%)	28 (39·4%)	2 (40·0%)	67 (15·7%)	8 (25·0%)	45 (30·8%)	4 (16·7%)
Lineage 3	19 (2·7%)	4 (5·6%)	1 (20·0%)	13 (3·1%)	0	1 (0·7%)	0
Lineage 4	465 (66·1%)	27 (38·0%)	2 (40·0%)	298 (70·0%)	22 (68·8%)	96 (65·8%)	20 (83·3%)
Sublineage†							
1.1.2 East African Indian	2 (0·3%)	0	0	2 (0·5%)	0	0	0
1.1.3 East African Indian	29 (4·1%)	8 (11·3%)	0	19 (4·5%)	0	2 (1·4%)	0
1.2.2 East African Indian	35 (5·0%)	4 (5·6%)	0	27 (6·3%)	2 (6·3%)	2 (1·4%)	0
2.2.1 Beijing	43 (6·1%)	7 (9·9%)	0	30 (7·0%)	0	6 (4·1%)	0
2.2.1 Beijing Asian African 1	8 (1·1%)	2 (2·8%)	0	5 (1·2%)	1 (3·1%)	0	0
2.2.1 Beijing Asian African 2	55 (7·8%)	13 (18·3%)	2 (40·0%)	29 (6·8%)	7 (21·9%)	4 (2·7%)	0
2.2.1.1 Beijing Pacific RD150	6 (0·9%)	6 (8·5%)	0	0	0	0	0
2.2.2 Beijing ancestral 1	42 (6·0%)	0	0	3 (0·7%)	0	35 (24·0%)	4 (16·7%)
3 Delhi-Central Asian Strain	10 (1·4%)	3 (4·2%)	1 (20·0%)	5 (1·2%)	0	1 (0·7%)	0
3.1.1 Delhi-Central Asian Strain	7 (1·0%)	1 (1·4%)	0	6 (1·4%)	0	0	0
3.1.2.1 Delhi-Central Asian Strain	2 (0·3%)	0	0	2 (0·5%)	0	0	0
4.1.1 X-type	1 (0·1%)	0	0	1 (0·2%)	0	0	0
4.1.1.1 X-type	13 (1·8%)	1 (1·4%)	0	9 (2·1%)	0	3 (2·1%)	0
4.1.1.3 X-type	49 (7·0%)	2 (2·8%)	0	19 (4·5%)	0	27 (18·5%)	1 (4·2%)
4.1.2 Euro-American	35 (5·0%)	1 (1·4%)	0	32 (7·5%)	0	2 (1·4%)	0
4.1.2.1 Haarlem	78 (11·1%)	0	0	49 (11·5%)	4 (12·5%)	25 (17·1%)	0
4.3.2 Latin-American Mediterranean	23 (3·3%)	0	0	21 (4·9%)	0	0	2 (8·3%)
4.3.2.1 Latin-American Mediterranean	7 (1·0%)	2 (2·8%)	0	5 (1·2%)	0	0	0
4.3.3 Latin-American Mediterranean	35 (5·0%)	1 (1·4%)	0	16 (3·8%)	5 (15·6%)	12 (8·2%)	1 (4·2%)
4.3.4.1 Latin-American Mediterranean	21 (3·0%)	1 (1·4%)	0	15 (3·5%)	0	3 (2·1%)	2 (8·3%)
4.3.4.2 Latin-American Mediterranean	7 (1·0%)	3 (4·2%)	0	4 (0·9%)	0	0	0
4.3.4.2.1 Latin-American Mediterranean	83 (11·8%)	5 (7·0%)	2 (40·0%)	58 (13·6%)	3 (9·4%)	14 (9·6%)	1 (4·2%)
4.4.1.1 S-type	63 (8·9%)	3 (4·2%)	0	32 (7·5%)	10 (31·3%)	5 (3·4%)	13 (54·2%)
4.4.2 Euro-American	2 (0·3%)	2 (2·8%)	0	0	0	0	0
4.6.2.2 Cameroon	2 (0·3%)	0	0	2 (0·5%)	0	0	0
4.7	3 (0·4%)	0	0	3 (0·7%)	0	0	0
4.8	29 (4·1%)	4 (5·6%)	0	22 (5·2%)	0	3 (2·1%)	0
4.9 H37Rv-like	14 (2·0%)	2 (2·8%)	0	10 (2·3%)	0	2 (1·4%)	0

Data are n (%). *The dataset total is the denominator used for calculating the percentages in this row. †The number in the top cell of each column is the denominator used for calculating the percentages for lineages and sublineages.

Table 1: **Genome-based drug resistance classification stratified by lineage and sublineage**

outbreak clusters most recent common ancestors we implemented a strict molecular clock with a Hasegawa-Kishino-Yano substitution model, and a gamma distributed among-site rate variation with four rate categories. Further details of these methods are in appendix 2 (p 2).

Statistical analysis

All statistical data analysis was performed using R (version 4.2.1). We performed one sample *t*-test to compare the age distribution, χ^2 test to compare the regional distribution, and the exact binomial test to compare treatment history and sex ratios. To test for trends over time in treatment history and sex composition we used the Pearson's χ^2 test. Linear regression analysis of bedaquiline and fluoroquinolone resistance frequency over time was performed with the Ggpmisc R package. We calculated frequencies of bedaquiline-resistant samples, bedaquiline-resistant samples with an *Rv0678* Ile491Phe mutation, bedaquiline-resistant samples without an *Rv0678* Ile491Phe mutation per year, and fluoroquinolone-resistant samples. We then applied linear regression to those values to determine if there is a significant

increase of resistance frequency in any of those four groups over time. Further details are in appendix 2 (p 2).

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

2606 Mtbc isolates submitted between Jan 1, 2015, and July 31, 2021, with an isoniazid or rifampicin resistance record, or both, were identified in the NTRL biobank. Of those, 1483 (56·9%) were from men, 1114 (42·7%) from women, and nine (0·4%) were unknown. 943 (36·2%) were considered new untreated cases, and 1574 (60·4%) as previously treated per WHO criteria (appendix 2 pp 10–12; appendix 3).[20] Culture and DNA extraction was successful for 964 Mtbc strains and WGS was successfully performed on 809 isolates (appendix 2 p 4). Details of strains successfully sequenced and those which were not sequenced are in appendix 2 (p 10). There were differences in sequencing success by province and year. Sequencing success ranged between 6·7 and 26·5% in samples from 2015 to 2018 compared with 38·2–66·7% in samples from 2019 to 2021.

To test for potential selection bias, we compared age, sex, and new or previously treated case distributions in sequenced and all samples. Although we did not detect differences for sex distribution (appendix 2 p 13), we found a difference in age distribution for year 2021 (p=0·046; appendix 2 p 11), and new or previously treated tuberculosis distribution in year 2016 (p=0·011; appendix 2 p 13). Then, we tested for changes in the composition of sequenced samples over time. The only significant change found was for treatment history which exhibited temporal variation, with lower percentages of new infections in 2018 (27%) and 2019 (33%) compared with the remaining years (40–48%; χ^2 test p=0·001; appendix 2 p 13).

Genome-based drug-resistant prediction classified 704 Mtbc strains as rifampicin resistant, which were included in further analysis (table 1; appendix 1). The strains have been further classified as rifampicin resistant (71 [10·1%]), rifampicin resistant and bedaquiline resistant (five [0·7%]), MDR (426 [60·5%]), MDR and bedaquiline resistant (32 [4·5%]), pre-XDR (146 [20·7%]), and XDR (24 [3·4%]) according to the 2021 WHO definitions (table 1). Interestingly, 61 (8·7%) of the 704 Mtbc strains investigated had evidence of bedaquiline resistance, most frequently due to mutations in *Rv0678*, while only one strain had a mutation in *atpE* (appendix 1). There were 29 unique mutations in *Rv0678*, of which all 15 SNPs, four (33%) of 12 indels, and one (50%) of two stop codons have been reported before, summing up to 69% of the detected bedaquiline or clofazimine resistance mutations. Besides in the 24 XDR Mtbc strains, bedaquiline resistance was found in five rifampicin-resistant strains and 32 MDR strains (table 1; appendix 1), indicating that bedaquiline resistance emerged independently from fluoroquinolone resistance. Additionally, prevalence of bedaquiline resistance increased significantly over time from 3% in 2016 to 14% in 2021 while remaining relatively stable for fluoroquinolone (figure 1). This trend was also seen when samples were stratified by treatment type (in new tuberculosis infections, 4% bedaquiline resistance in years 2015–19 and 15% in years 2020–21; in retreatment tuberculosis, 5% bedaquiline resistance in years 2015–19 and 13% in years 2020–21; appendix 2 p 14). A high proportion of bedaquiline-resistant strains (24 [39·3%] of 61) carried the *rpoB* Ile491Phe mutation, 23 of which were closely linked to the diagnostic escape Eswatini outbreak strain (group_56; figure 2, 3).[8]

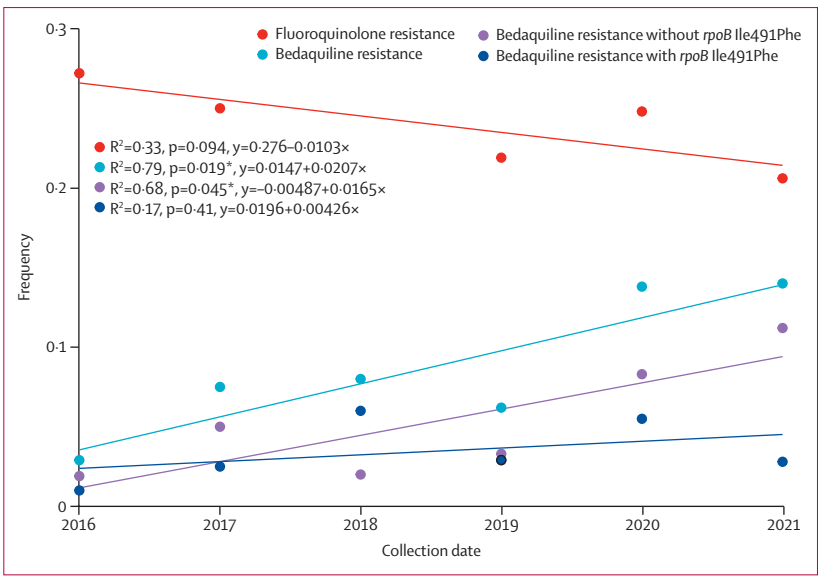


Figure 1: **Frequency of bedaquiline and fluoroquinolone resistance in *Mycobacterium tuberculosis* complex strains over time**

Linear regression analysis of correlation of bedaquiline and fluoroquinolone resistance frequency with the year of strain isolation, additionally stratified by presence or absence of the *rpoB* Ile491Phe mutation for bedaquiline. Frequencies of resistant strains were calculated over the period 2016–21.

See **Online** for appendix 3

To investigate possible correlations between Mtbc strain type and drug-resistant transmission dynamics, we classified the strains investigated into main phylogenetic lineages using canonical SNPs as described previously (table 1; appendix 1).[16,17]

Of the 704 strains, 465 strains (66·1%) belonged to lineage 4, 154 to lineage 2 (21·9%), 66 to lineage 1 (9·4%), and 19 to lineage 3 (2·7%). Lineage 2 strains were classified into the following sublineages: 2.2.1 Beijing (n=43), 2.2.1 Beijing Asian African 1 (n=8), 2.2.1 Beijing Asian African 2 (n=55), 2.2.1.1 Beijing Pacific RD150 (n=6), and 2.2.2 Beijing ancestral 1 (n=42). Lineage 4 strains were classified into 17 sub-lineages (table 1; appendix 1), with strains of 4.3.4.2.1 Latin-American Mediterranean (n=83), 4.4.1.1 S-type (n=63), 4.1.2 Euro-American (n=35), 4.3.3 Latin-American Mediterranean (n=35), and 4.1.1.3 X-type (n=49) being most prominent.

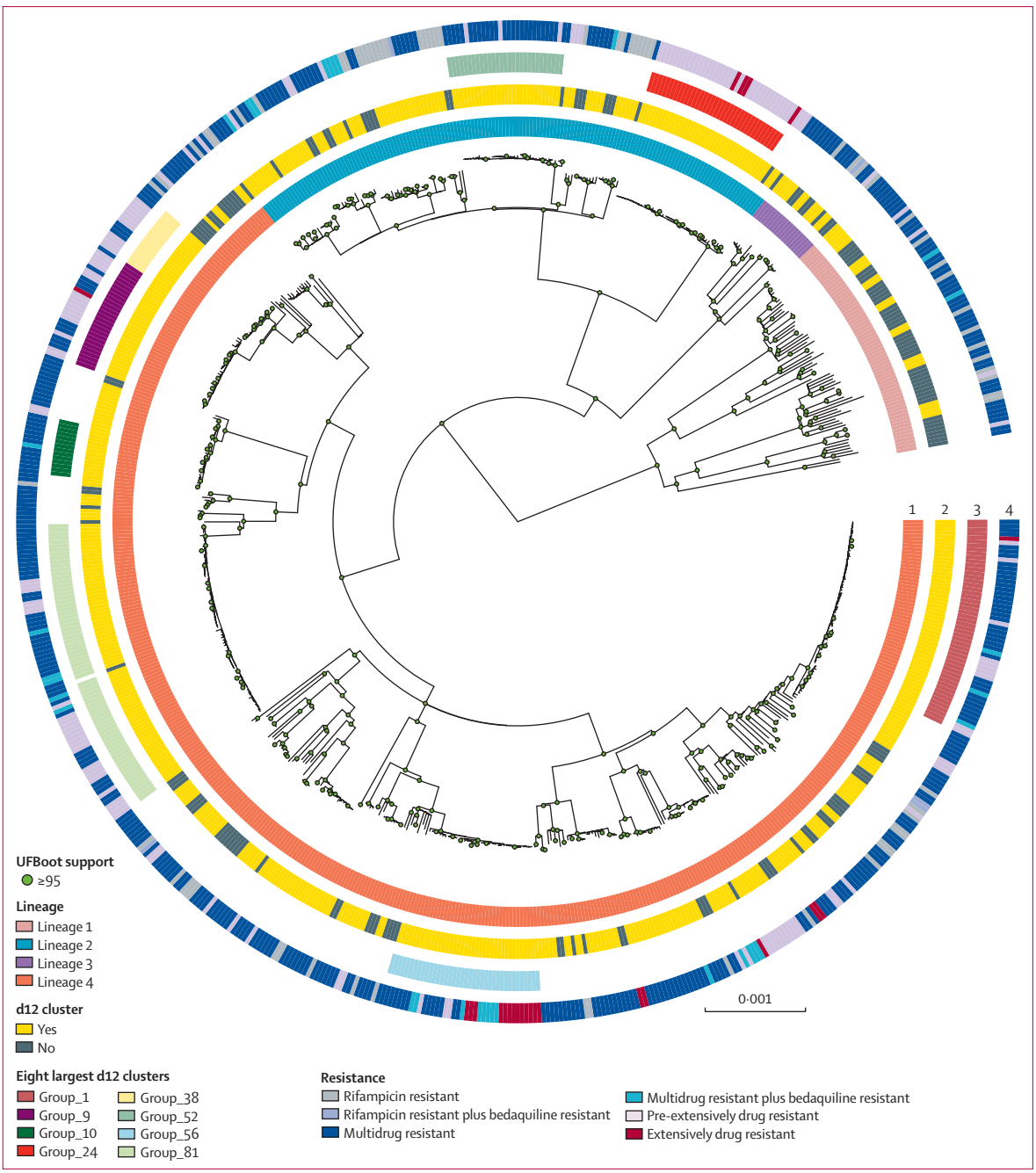
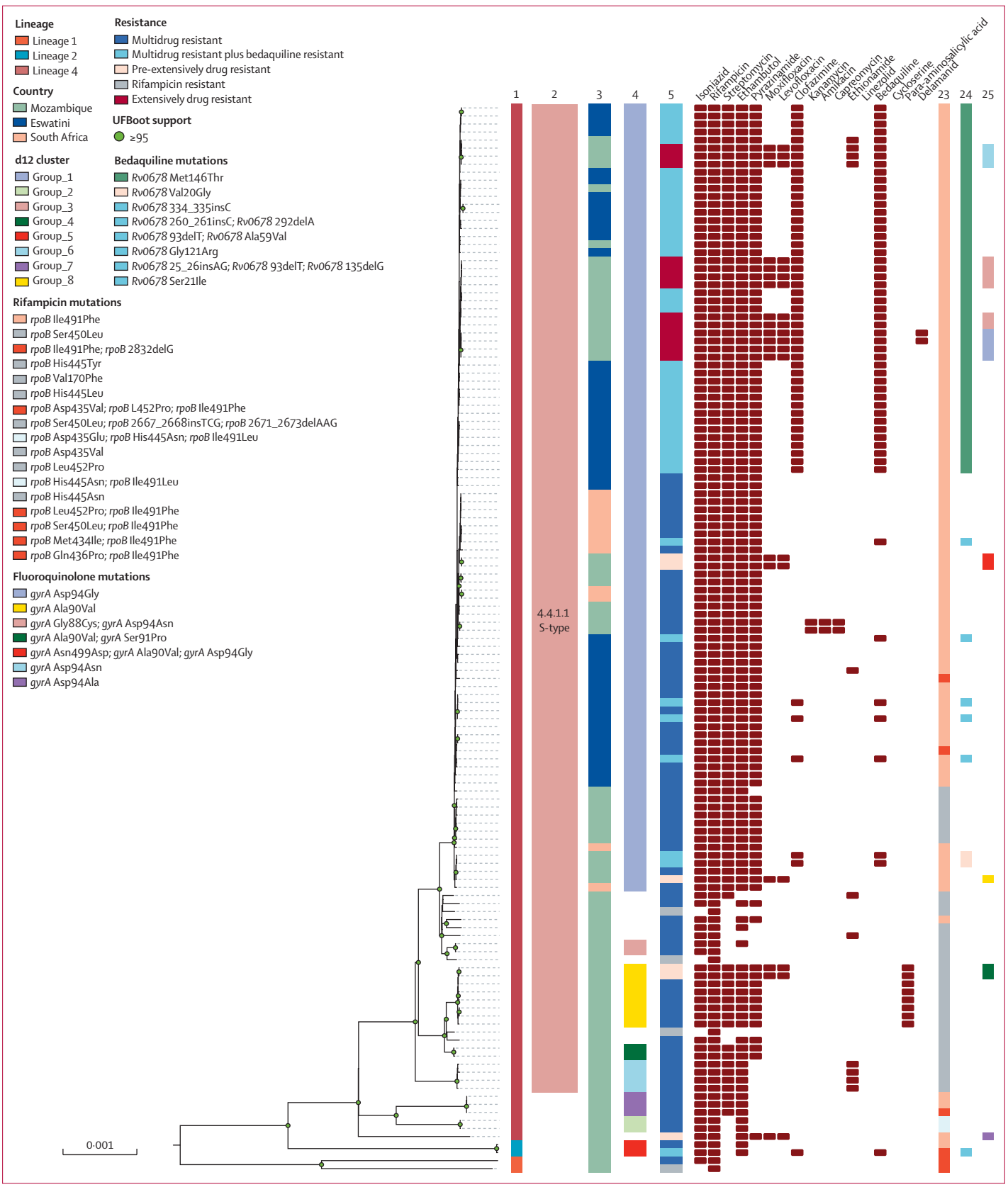


Figure 2: **Phylogeny, cluster groups, and resistance types of the 704 rifampicin-resistant *Mycobacterium tuberculosis* complex strains**
The maximum likelihood phylogenetic tree was calculated from the concatenated SNP alignment of 18 630 informative sites (appendix 2 p 2). Circle 1 shows *M tuberculosis* complex strain lineage; circle 2 shows clustering status based on a single-nucleotide polymorphism distance of 12; circle 3 shows the eight largest clusters of the dataset; and circle 4 shows resistance type determined by genomic resistance prediction.

In a broader classification, Latin-American Mediterranean strains represented most of the lineage 4 strains (176 [37·8%] of 465), followed by strains of the lineages Haarlem (78 [16·8%]), X-type (63 [13·5%]), and S-type (63 [13·5%]; appendix 1). The phylogenetic strain classification was fully consistent with strains' position in the maximum likelihood tree and a minimum spanning tree calculated based on a total of 18 630 informative sites differentiating any of the 704 Mtbc strains (figure 2; appendix 2 pp 5–6).

A cluster analysis was performed to investigate recent transmission. Based on a maximum distance of 12 SNPs, 557 Mtbc strains (79%) were grouped into 86 clusters ranging in size from 2 to 72 isolates (appendix 1; figure 2,


Lineage
Lineage 1
Lineage 2
Lineage 4
Country
Mozambique
Eswatini
South Africa
d12 cluster
Group_1
Group_2
Group_3
Group_4
Group_5
Group_6
Group_7
Group_8
Resistance
Multidrug resistant
Multidrug resistant plus bedaquiline resistant
Pre-extensively drug resistant
Rifampicin resistant
Extensively drug resistant
UFBoot support
≥95
Bedaquiline mutations
Rv0678 Met146Thr
Rv0678 Val20Gly
Rv0678 334_335insC
Rv0678 260_261insC; Rv0678 292delA
Rv0678 93delT; Rv0678 Ala59Val
Rv0678 Gly121Arg
Rv0678 25_26insAG; Rv0678 93delT; Rv0678 135delG
Rv0678 Ser21Ile
Rifampicin mutations
rpoB Ile491Phe
rpoB Ser450Leu
rpoB Ile491Phe; rpoB 2832delG
rpoB His445Tyr
rpoB Val170Phe
rpoB His445Leu
rpoB Asp435Val; rpoB L452Pro; rpoB Ile491Phe
rpoB Ser450Leu; rpoB 2667_2668insTCG; rpoB 2671_2673delAAG
rpoB Asp435Glu; rpoB His445Asn; rpoB Ile491Leu
rpoB Asp435Val
rpoB Leu452Pro
rpoB His445Asn; rpoB Ile491Leu
rpoB His445Asn
rpoB Leu452Pro; rpoB Ile491Phe
rpoB Ser450Leu; rpoB Ile491Phe
rpoB Met434Ile; rpoB Ile491Phe
rpoB Gln436Pro; rpoB Ile491Phe
Fluoroquinolone mutations
gyrA Asp94Gly
gyrA Ala90Val
gyrA Gly88Cys; gyrA Asp94Asn
gyrA Ala90Val; gyrA Ser91Pro
gyrA Asn499Asp; gyrA Ala90Val; gyrA Asp94Gly
gyrA Asp94Asn
gyrA Asp94Ala
Isoniazid
Rifampicin
Streptomycin
Ethambutol
Pyrazinamide
Moxifloxacin
Levofloxacin
Clofazimine
Kanamycin
Amikacin
Capreomycin
Ethionamide
Linezolid
Bedaquiline
Cycloserine
Para-aminosalicylic acid
Delamanid
1
2
3
4
5
23
24
25
4.4.1.1
S-type
0·001

appendix 2 p 7). The eight largest clusters, group_10 (n=14), group_38 (n=16), group_9 (n=28), group_52 (n=29), group_24 (n=36), group_56 (n=38), group_1 (n=52), and group_81 (n=72), represented 41% of the 704 Mtbc strains investigated (table 2). The proportion of clustered strains increased from 42% among rifampicin resistant to 78% among MDR, 94% among MDR plus bedaquiline resistant, 94% among pre-XDR, and 96% among XDR strains, pointing towards increasing transmission by drug-resistant category (table 2). Of note, 31 of the 38 group_56 strains carry the Ile491Phe mutation (appendix 1; figure 3).

Clustering among bedaquiline-resistant strains was also high (58 [95%] of 61; appendix 1). Indeed, more than two-thirds (n=45) of the 58 clustered bedaquiline-resistant strains shared an identical *Rv0678* mutation with another strain in a genomic cluster indicating recent transmission. The number of strains with the same *Rv0678* mutation in a cluster ranged from two strains to 19 strains in cluster group_56 with the *Rv0678* Met146Thr mutation, which in addition carried the Ile491Phe *rpoB* mutation (appendix 1; appendix 2 p 7). Interestingly, primary transmission of bedaquiline resistance was also confirmed for rifampicin resistant, and MDR strains (appendix 1).

None of the circulating clustered strains had reached dominance on a population level. However, eight main clonal clusters comprising 14 to 72 strains were identified from the tree topology (figure 2; appendix 2 p 7; table 2). All strains of these eight main d12 clusters were at least MDR, with four clusters including a high proportion of pre-XDR or XDR resistance genotypes (table 2). Overall, the strains of the eight largest clusters accounted for 69% of the pre-XDR and 79% of the XDR strains (table 2). All strains of the aforementioned clusters acquired resistance to some or all first-line drugs (appendix 1). According to the Bayesian analyses, these circulating clusters are relatively recent (appendix 2 p 9), and the time to their most common ancestors was dated between 2000 and 2010. The youngest clonal cluster was group_52 and the oldest, group_56, potentially linked to longer cross-border transmission in the region.

Figure 3: **Phylogeny, cluster groups, and resistance types of 133 Mtbc strains**
In this analysis, all 4.4.1.1 S-type strains of this study (n=63) were combined with 4.4.1.1 S-type strains (positive for *rpoB* Ile491Phe) from Eswatini (n=48) and South Africa (n=12). In addition, ten additional Mtbc strains from this study with an amino acid change at codon 491 in *rpoB* were included. Maximum likelihood phylogenetic tree built from the concatenated SNP alignment of 4349 informative sites (appendix 2 p 2). Track 1 shows the Mtbc lineage; track 2 shows the Mtbc sublineage only for 4.4.1.1; track 3 shows the country of origin; track 4 shows the clustered groups based on an SNP distance of 12; track 5 shows the resistance type determined by genomic resistance prediction; tracks 6–22 show the absence or presence of resistance to the indicated antibiotic; track 23 shows specific *rpoB* mutation; track 24 shows specific *Rv0678* mutation; and track 25 shows specific *gyrA/B* mutation. Mtbc=*Mycobacterium tuberculosis* complex. SNP=single-nucleotide polymorphism.

A more detailed phylogenetic analysis on cluster level (appendix 2 pp 5–8) revealed that all group_24 strains (2.2.2 Beijing ancestral 1) had evidence of fluoroquinolone resistance and four also had bedaquiline resistance (appendix 1; appendix 2 p 8). 31 of 38 group_56 strains belonged to an S-type outbreak strain previously identified in Eswatini and South Africa carrying the *rpoB* Ile491Phe mutation.[8,21] In addition, 19 of them had the *Rv0678* Met146Thr mutation, two the *Rv0678* Val20Gly and another two the *Rv0678* Leu117Arg mutation, all of which confer phenotypic bedaquiline resistance (appendix 2 p 8).[8] Among the 19 strains with *Rv0678* Met146Thr mutation 13 had acquired fluoroquinolone resistance mutation making them XDR (table 1; appendix 2 p 8) and two even had an additional delamanid resistance mutation (appendix 1). Overall, ten (31·3%) of the 32 MDR plus bedaquiline-resistant strains and 13 (54·2%) of the 24 XDR strains in the study population belonged to group_56.

A combined phylogeny was calculated for Ile491Phe strains from Eswatini (n=48),[8] South Africa (n=12),[21] and Mozambique (n=63; figure 3). The phylogeny revealed a very close relationship of Ile491Phe strains from all countries, that applied for different subvariants—eg, with and without bedaquiline resistance mutation. Interestingly, while the Ile491Phe variant with the main bedaquiline resistance mutation Met146Thr occurred in Eswatini and Mozambique, additional fluoroquinolone resistance was only found in strains from Mozambique (figure 3).

Of note, the *Rv0678* Ile491Phe mutation and other Ile491 mutations also occurred in 11 Mtbc strains not belonging to the group_56, indicating homoplastic evolution of rifampicin molecular diagnostic escape strains (figure 3; appendix 1).

Discussion

This is the first study employing high resolution WGS to investigate drug resistance and clustering of rifampicin-resistant Mtbc strains collected in Mozambique over a 5-year period. We found that one in four rifampicin-resistant Mtbc strains had mutations conferring fluoroquinolone resistance and that bedaquiline resistance was increasing from 3% in 2016 to 14% in 2021. Such high levels of resistance to fluoroquinolone and bedaquiline are likely to jeopardise national rollout of the new WHO approved 6 months regimens of bedaquiline, pretomanid, linezolid, and moxifloxacin or bedaquiline, pretomanid, and linezolid.[22] Although the identification of strains with mutations conferring rifampicin resistance outside of the *rpoB* hotspot region, specifically Ile491Phe, have been reported from Eswatini and South Africa,[8,21,23] they have not yet been reported from other countries in the region. Our data show that a high proportion of MDR plus bedaquiline resistant, and XDR Mtbc strains in Mozambique are due to strains with the Ile491Phe mutation, which remain undetected by Xpert MTB/RIF and commercially available line probe

	Sublineage	Total	Rifampicin resistant	Rifampicin resistant plus bedaquiline resistant	Multidrug resistant	Multidrug resistant plus bedaquiline resistant	Pre-extensively drug resistant	Extensively drug resistant
Dataset*	NA	704 (100%)	71 (10·1%)	5 (0·7%)	426 (60·5%)	32 (4·5%)	146 (20·7%)	24 (3·4%)
Clustered d5†								
Yes	NA	478 (67·9%)	26 (36·6%)	5 (100%)	277 (65·0%)	28 (87·5%)	120 (82·2%)	22 (91·7%)
No	NA	226 (32·1%)	45 (63·4%)	0	149 (35·0%)	4 (12·5%)	26 (17·8%)	2 (8·3%)
Clustered d12†								
Yes	NA	557 (79·1%)	30 (42·3%)	5 (100%)	332 (77·9%)	30 (93·8%)	137 (93·8%)	23 (95·8%)
No	NA	147 (20·9%)	41 (57·7%)	0	94 (22·1%)	2 (6·3%)	9 (6·2%)	1 (4·2%)
Eight largest clusters†		285 (40·5%)	0	0	149 (35%)	17 (53·1%)	100 (68·5%)	19 (79·2%)
Group_81	4.1.2.1 Haarlem	72 (10·2%)	0	0	43 (10·1%)	4 (12·5%)	25 (17·1%)	0
Group_1	4.3.4.2.1 Latin-American Mediterranean	52 (7·4%)	0	0	38 (8·9%)	3 (9·4%)	10 (6·8%)	1 (4·2%)
Group_56	4.4.1.1 S-type	38 (5·4%)	0	0	12 (2·8%)	10 (31·3%)	3 (2·1%)	13 (54·2%)
Group_24	2.2.2 Beijing ancestral 1	36 (5·1%)	0	0	0	0	32 (21·9%)	4 (16·7%)
Group_52	2.2.1 Beijing	29 (4·1%)	0	0	26 (6·1%)	0	3 (2·1%)	0
Group_9	4.1.1.3 X-type	28 (4·0%)	0	0	11 (2·6%)	0	16 (11·0%)	1 (4·2%)
Group_38	4.1.1.3 X-type	16 (2·3%)	0	0	5 (1·2%)	0	11 (7·5%)	0
Group_10	4.1.2 Euro-American	14 (2·0%)	0	0	14 (3·3%)	0	0	0

Data are n (%). NA=not applicable. *The dataset total is the denominator used for calculating the percentages in this row. †The number in the top cell of each column is the denominator used for calculating the percentages for clusters.

Table 2: **Genome-based drug resistance classification stratified by cluster**

assays, as none of these carried an additional canonical *rpoB* mutation (appendix 1). Additionally, we found a high cluster rate of 79% among MDR strains, 94% among pre-XDR strains, and 96% among XDR strains suggesting transmission as main driver. High level of clustering among bedaquiline-resistant strains and the fact that two-thirds of clustered bedaquiline-resistant strains share the same *Rv0678* mutation point towards ongoing transmission as one of the major drivers for bedaquiline resistance.

The prevalence of fluoroquinolone resistance among rifampicin-resistant Mtbc strains in most southern African countries, except for South Africa, is less than 10%.[1] Hence, the fluoroquinolone resistance prevalence of 23% among MDR strains from Mozambique investigated in our study is high compared with other countries in the region. Our data indicate that a possible reason for this is the ongoing transmission of fluoroquinolone-resistant strains potentially driven by such as the group_24 strains.

In addition to high prevalence of fluoroquinolone resistance, our data show an increase of bedaquiline resistance from 3% in 2016 to 14% in 2021 due to acquisition of *Rv0678* mutations and recent transmission of bedaquiline-resistant Mtbc strains. Emergence of bedaquiline resistance was also observed in South Africa, and other settings albeit at much lower levels.[4–7,24] In South Africa, bedaquiline resistance was also largely due to *Rv0678* mutations and has been associated with treatment failure and amplification of additional drug resistance.[5,7]

The fact that bedaquiline resistance in Mozambique was not limited to fluoroquinolone-resistant strains, but also observed in rifampicin resistant, and MDR strains, might suggest that current MDR tuberculosis treatment regimens are unable to prevent development of bedaquiline resistance on a population level. The pattern of resistance emergence observed in outbreak strains implies a stepwise evolution from pre-existing bedaquiline or fluoroquinolone resistances towards XDR. Our data underline a high risk of fluoroquinolone resistance development in people infected with Ile491Phe outbreak strains with bedaquiline resistance that have been detected in this study and in Eswatini.[8] These data highlight the urgent need to implement effective rapid genotypic drug susceptibility tests (ie, targeted sequencing from sputum or other materials)[25,26] to ensure efficacy and longevity of the newly endorsed regimens for treatment of drug-resistant tuberculosis including short course bedaquiline, pretomanid, linezolid, and moxifloxacin, or bedaquiline, pretomanid, and linezolid regimens.[22] Also, in contrast to other studies in the region,[27] the proportion of rifampicin-resistant Mtbc strains belonging to lineage 1, which have an intrinsically higher pretomanid minimal inhibitory concentration,[28] was relatively high, having potential consequences for applying regimens containing this drug especially in combination with high fluoroquinolone and bedaquiline resistance rates.

Despite only including 30% of Mtbc strains submitted to the NTRL over a 7-year period, we found a high cluster rate among the investigated strains indicating intense

transmission of rifampicin resistance, MDR, pre-XDR, and XDR. Several large clusters comprising up to 72 Mtbc strains (group_81, 4.1.2.1 Haarlem) were identified. These outbreak clones made up a large proportion of MDR, MDR plus bedaquiline, pre-XDR, and XDR Mtbc strains. Similar levels of clustering among drug-resistant strains have been reported from India, across central Asia and in Eswatini and South Africa, showing the effect of the transmission and dominant drug-resistant clones on the drug-resistant tuberculosis epidemic.[4,14,17,29]

Ongoing transmission of drug-resistant tuberculosis might be a result of underdiagnosis or ineffective treatment, or both. Indeed, the diagnostic gap for rifampicin-resistant tuberculosis is huge in Mozambique with an estimated 75% of rifampicin-resistant tuberculosis remaining undiagnosed due to the limited number of laboratories and GeneXpert inefficient sample transport systems.[1,10] In addition, the spread of the diagnostic escape *rpoB* Ile491Phe clone not detected by conventional molecular drug-resistant diagnostic assays such as Xpert MTB/RIF and the line probe assay MTBDRplus challenges accurate diagnosis of rifampicin resistance with current diagnostic algorithms and tools.[8,21,23] The phylogenetic analysis combining strains from Eswatini, South Africa, and Mozambique shows the close genetic relationship of the Ile491Phe strains investigated and points towards cross border migration and transmission. People with Mtbc Ile491Phe strains are unlikely to be diagnosed with rifampicin-resistant tuberculosis, resulting in inadequate treatment with first-line tuberculosis drugs, treatment failure, and ongoing transmission.[8,23] Even among people with failed tuberculosis treatment, accurate diagnosis of rifampicin resistance, MDR, or XDR is likely to be delayed because in the absence of sequencing capacity, time-consuming phenotypic drug susceptibility tests are required. A high proportion of Ile491Phe strains were resistant to all first-line drugs and had additional resistance to bedaquiline, fluoroquinolone, and delamanid, suggesting that people with Ile491Phe associated tuberculosis or those who they acquired it from had received multiple ineffective treatment regimens. These observations are in line with a recent study in KwaZulu-Natal, South Africa, that showed that Ile491Phe strains are the main reason for incorrectly diagnosing rifampicin-resistant tuberculosis as drug-susceptible tuberculosis leading to delayed diagnostics and resistance accumulation.[23]

Our study has clear limitations. First, only samples sent to the NTRL were included in the study and, thus, results cannot be generalised beyond the NTRL catchment areas. Also, while some provinces such as Tete, Mozambique, submit samples to the NTRL, the geographical distance and transport challenges limit the numbers of samples sent from these areas to relatively few. Furthermore, fewer samples were submitted for testing to the NTRL in more recent years (2020 and 2021) compared with the years 2016–19 probably due to the COVID-19 pandemic, which resulted in fewer MDR tuberculosis diagnoses, reduced laboratory capacity, and limited transport options due to national lockdowns. Hence, any differences over time need to be interpreted with caution as confounding by time and selection bias cannot be ruled out. WGS was performed on a third of drug-resistant strains identified in the biobank at the NTRL in Maputo, Mozambique. Two-thirds could not be sequenced due to unsucessful subculture or low quality DNA post extraction. Successful sequencing was more probable for strains submitted more recently (2019–21), which needs to be accounted for when interpreting the changes over time. Unfortunately, reasons for submitting a sample—ie, rifampicin resistance diagnosed by Xpert MTB/RIF, smear positivity at 2 months, drug-susceptible or drug-resistant treatment, treatment failure, or relapse—were not available. This missing information potentially led to undetected oversampling of samples of some submission categories, which might have influenced the resistance rates observed in our study.

The rate of rifampicin-resistant tuberculosis caused by Mtbc strains with mutations outside of the *rpoB* hotspot region, specifically Ile491Phe, is likely to be underestimated as the NTRL receives primarily samples from people diagnosed with rifampicin-resistant tuberculosis using the GeneXpert assay. Also, we did not have any clinical or epidemiological data, which would have allowed more detailed analysis specifically investigating epidemiological links between clustered isolates. The strengths of the study include the large sample size, and that strains were collected over a 7-year period from all provinces of Mozambique.

In conclusion, the high prevalence of fluoroquinolone resistance, increasing prevalence of bedaquiline resistance, and substantial number of rifampicin-resistant strains with Ile491Phe mutations in combination with high clustering rates requires an effective public health intervention to ensure the drug-resistant tuberculosis epidemic in Mozambique and the region is not spiralling out of control. Enhanced diagnosis of people with rifampicin-resistant tuberculosis is urgently needed to close the diagnostic gap using the existing diagnostic tools but potentially paired with novel strategies such as a focus on cross border travel and groups at high risk. In addition, considering the limitations of this study, well designed prospective studies are needed to verify our findings and to understand how best to identify those people infected with a rifampicin-resistant Ile491Phe Mtbc strain, risk groups, and transmission or resistance development factors. Given that Ile491Phe mutations often occur together with isoniazid resistance, routine diagnosis of isoniazid resistance could trigger either phenotypic rifampicin drug susceptibility tests or targeted sequencing.[25,26] Importantly, continuous high quality surveillance using either WGS or targeted sequencing is needed to monitor the evolving bedaquiline and fluoroquinolone resistance,

as these jeopardise the new and old short course oral regimens. Such surveillance should not be confined to rifampicin-resistant tuberculosis only, and should include a proportion of representative drug susceptible strains.

Contributors

IB, TF, CU, CA, TN, NI, KK, LdA, TW, SN, and SV conceived the idea and designed the study, and analysed and interpreted the data. IB, CU, and SN directly accessed and verified the underlying data reported in the manuscript. All authors had full access to all the data in the study. All authors contributed to obtaining and assembling the data. IB, TF, CU, KK, SV, and SN wrote the initial draft of the paper. All authors contributed to data interpretation and the final draft of the paper and approved the final version of the manuscript.

Declaration of interests

We declare no competing interests.

Data sharing

Sequencing data in FASTQ format can be downloaded from the European Nucleotide Archive at https://www.ebi.ac.uk/ena/browser/home (accession number PRJEB62665). Data sources are listed in the Article, appendix 1, and appendix 3.

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