Transmission of pre-XDR and XDR-TB in the Mumbai Metropolitan Region, India

Viola Dreyer[&,1,5], Ayan Mandal[2,&], Prachi Dev[2,&], Matthias Merker[1], Ivan Barilar[1], Christian Utpatel[1], Kayzad Nilgiriwala[2], Camilla Rodrigues[3], Derrick W. Crook[4], the CRyPTIC Consortium[5], Nerges Mistry[2,$], Stefan Niemann[$,1,6]

[1]Molecular and Experimental Mycobacteriology, Research Center Borstel, Germany

[2]The Foundation for Medical Research, Dr. Kantilal J. Sheth Memorial Building, 84-A, R. G. Thadani Marg, Worli, Mumbai - 400 018, India

[3]Department of Microbiology, P. D. Hinduja National Hospital and Medical Research Centre, Mumbai, India

[4]Nuffield Department of Medicine, University of Oxford, Oxford, UK.

[5]CRyPTIC Consortium. Author list in supplement.

[6]German Center for Infection Research (DZIF), Partner Site Hamburg-Lübeck-Borstel-Riems, Germany;

[&]equal contribution

[$]equal contribution

Corresponding author: Stefan Niemann, Molecular and Experimental Mycobacteriology, Forschungszentrum Borstel, Parkallee 1, D-23845 Borstel, Germany. Phone: (49) 4537 1887620; Fax: (49) 4537 188 -2091 E-mail: sniemann@fz-borstel.de.

Abstract

Background. Multidrug-resistant (MDR) and extensively drug resistant (XDR) *Mycobacterium tuberculosis* complex (MTBC) strains are a great challenge for tuberculosis (TB) control in India. Still, factors driving the MDR/XDR epidemic in India are not well defined.

Methods. Whole genome sequencing (WGS) data from 1 852 MTBC strains obtained from patients from a tertiary care hospital laboratory in Mumbai were used for phylogenetic strain classification, resistance prediction, and cluster analysis (12 allele distance threshold). Factors associated with pre-XDR/XDR-TB were defined by odds ratios and a multivariate logistic regression model.

Results. 1 017 MTBC strains were MDR, out of which 57.8 % (n=591) were pre-XDR, and 17.9 % (n=183) were XDR. Lineage 2 (L2) strains represented 41.7 % of the MDR, 77.2 % of the pre-XDR, and 86.3 % of the XDR strains, and were significantly associated with pre-XDR/XDR-TB ($P < 0.001$). Cluster rates were high among MDR (78 %) and pre-XDR/XDR (85 %) strains with three dominant L2 strain clusters (Cl 1-3) representing half of the pre-XDR and two thirds of the XDR-TB cases. Cl 1 strains accounted for 52.5 % of the XDR MTBC strains. Transmission could be confirmed by identical mutation patterns of particular pre-XDR/XDR strains.

Conclusions. High rates of pre-XDR/XDR strains among MDR-TB patients require rapid changes in treatment and control strategies. Transmission of particular pre-XDR/XDR L2 strains is the main driver of the pre-XDR/XDR-TB epidemic. Accordingly, control of the epidemic in the region requires measures with stopping transmission esp. of pre-XDR/XDR L2 strains.

Funding source: Parts of this work have been supported within the CRYPTIC consortium by the Bill & Melinda Gates Foundation [OPP1133541] and the Wellcome Trust [200205/Z/15/Z], the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germanys Excellence Strategy – EXC 2167 Precision Medicine in Inflammation, and the Leibniz Science Campus Evolutionary Medicine of the LUNG (EvoLUNG). The funders had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication**.**

Research in context

Evidence before this study

India is particularly affected by multidrug resistant (MDR), and extremely drug resistant (XDR) tuberculosis (TB), e.g., accounting for 27 % of the globally newly emerging MDR-TB cases. Over the past 2 decades several studies have highlighted high rates of drug resistance in Mumbai. More recently in the Mumbai metropolitan area, around 5,000 new MDR-TB cases are notified annually, with a more than 30% increase over a three-year period from 2015 to 2017. These data are extremely alarming, threatening human health and TB control in one of the most populated regions of the world. To tackle this dramatic situation, a better understanding of the epidemiological reasons is urgently needed to better guide TB control interventions. Small studies employing whole genome sequencing (WGS) of clinical *Mycobacterium tuberculosis* complex (MTBC) strains suggest that occurrence and transmission of particular MDR MTBC strain types may be important factors, however, large scale data on the determinants of the MDR/XDR-TB epidemic in the region are missing.

Added value of this study

To understand more precisely the reasons for the high MDR/XDR-TB rates, we performed WGS of 1 852 MTBC strains obtained from patients from a tertiary care hospital laboratory in Mumbai. We found a high rate of pre-XDR (57.8%) and XDR (17.9 %) among the 1017 MDR MTBC strains. Cluster rates were high among MDR (78 %) and pre-XDR/XDR (85 %) strains with three dominant L2 (Beijing) strain clusters (Cl 1-3) representing half of the pre-XDR and two thirds of the XDR-TB cases. Just cluster 1 strains accounted for 52.5 % of the XDR MTBC strains. Cluster 1-3 strains also had very high combined resistance rates to first line and second line drugs, thus, reducing available group A, B, and C drugs proposed for the treatment of MDR-TB cases to a minimal set and making the application of the short MDR-TB regimen impossible. Transmission could be further confirmed by identical mutation patterns of particular pre-XDR/XDR strains.

Implication of all the available evidence

Our study documents extremely high pre-XDR and XDR-TB levels in the MDR-TB strain population that has not been described before. This remarkable shift towards pre-XDR is mediated by high frequency FQ resistance mutations that, in combination with particular injectable drug resistance mutations, result in XDR-TB. In addition, pre-XDR, and XDR strains are virtually all L2 strains and show high cluster rates as an indication of ongoing transmission. Thus, successful control of the DR epidemic urgently requires measures stopping transmission of MDR/pre-XDR/XDR L2 strains in this region. As the majority of pre-XDR strains are already FQ resistant, treatment options are limited and urgent modification in treatment strategies are needed, for example, comprehensive resistance detection for

all cases accompanied by design of new effective treatment combinations. It is likely, that the uniform use of treatment regimens including newest MDR-TB drugs without precise knowledge of each case's resistance patterns combined with close patient monitoring likely results in ongoing transmission and further resistance development as described previously. In light of previously reported associations between BCG vaccine escape and rapid spread of L2 strains, vaccine non-responsiveness of MDR/pre-XDR/XDR L2 strains may prove to be a likely scenario in Mumbai.

Introduction

Multidrug-resistant (MDR) tuberculosis (TB) caused by *Mycobacterium tuberculosis* complex (MTBC) strains resistant to at least isoniazid (INH) and rifampicin (RMP) poses a great challenge to global TB control. More than 400 000 new MDR-TB cases are notified annually [1]; 50 % of these coming from India (27 %), China (14 %) and countries of the Russian Federation (9 %). This makes them the epi-centers of the current MDR-TB epidemic and key countries for the successful future intervention against MDR-TB[1]. Approx. 6 % of MDR-TB cases are already estimated to be extensively drug resistant (XDR, additional resistance to one of the fluoroquinolones [FQs] as well as to one of the injectable drugs, amikacin [AMI], capreomycin [CAP], or kanamycin [KAN])[2,3].

The treatment of MDR-TB patients is longer, based on less effective, more toxic drugs, and in 2019, the cure rate was only 57 % on a global level[1,3]. With 39 %, the treatment success rate is even lower for XDR-TB patients[2]. As ineffective treatment is an important factor driving transmission[4], the potential of MDR/XDR MTBC strains to transmit may be even higher compared to susceptible MTBC strains[5].

As outlined above, India is particularly affected and, thereby becomes one of the main drivers of the current MDR/XDR-TB epidemic. Few studies on MDR/XDR-TB in India underlines the urgent need to gain more and better knowledge of the genetics of resistance determinants, evolution and transmission, of MDR/XDR MTBC strains in the region[6,7]. Indeed, it is of particular importance to understand the origins and driving forces of the MDR/XDR-TB epidemic in the country including ongoing transmission of already highly resistant clones[8–10], but only few studies have used state-of-the-art whole genome sequencing (WGS) combined with epidemiological techniques to investigate transmission in India so far[11–14].

To address these knowledge gaps, we performed a retrospective genomic epidemiological analysis based on WGS of 2 040 MTBC strains mainly from the Mumbai metropolitan region, India. The strains were obtained from a tertiary care hospital laboratory in Mumbai, which providing comprehensive

drug susceptibility testing (DST) of MTBC strains. WGS data were used to determine MTBC-lineage, resistance to first- and second-line drugs, and transmission inference of MTBC strains based on sequence-based cluster analysis.

Methods

Study design

A total of 2 040 MTBC strains from patients were retrospectively collected as random sequential samples for the CRyPTIC Consortium Project between February 2017 and May 2018 (15 months) from the laboratory of a tertiary care hospital in India and WGS. Considering that in Mumbai around 5000 MDR cases are reported annually, the study covered approx. 16 % of MDR cases occurring in the region (n= 1017/6250) (source: https://portal.mcgm.gov.in/)[15]. One hundred and eighty-eight isolates were excluded from analysis due to sequence deficiencies. A majority (n = 1773) of the remaining isolates (n = 1852) were collected from the Mumbai Metropolitan region (MMR) of the western State of Maharashtra while 46 were derived from distal parts of Maharashtra and neighboring States/Union Territories and 33 from Delek Hospital, Himachal Pradesh, North India. Approval for the CRyPTIC study was obtained from the Health Ministry's Screening Committee (HMSC), Government of India dated 6th October 2016. Approval was also obtained from the Institutional Ethics Committee (IEC) of The Foundation for Medical Research, Mumbai (Ref nos. FMR/IEC/TB/01a/2015 and FMR/IEC/TB/01b/2015) and Institutional Review Board of P.D. Hinduja Hospital and Medical Research Centre, Mumbai (Ref no. 915-15-CR [MRC]).

Molecular methods

Genomic DNA was isolated from the 2 040 patient samples using FastPrep24 lysis method (MP Biomedicals, California, USA) as per standard protocol and quantified using Qubit (Life Technologies, Carlsbad, California, USA). Libraries for WGS were prepared using Nextera XT DNA Library Prep Kit and sequencing was performed on the Illumina NextSeq500 machine as per manufacturer's protocol

(Illumina Inc., San Diego, California, USA) producing 2 x 151 base pair reads. Fastq files are available at the European Nucleotide Archive (ENA, Table S1).

Genome analysis

All WGS data were analyzed using the MTBSeq pipeline (Version 1.0.3)[16]. Details are described in supplemental methods (Text S1). Phylogenetic lineages (MTBC-lineages and known Beijing subgroups) were inferred from specific SNPs based on Coll *et al.* 2014[17] and Merker *et al.* 2015[8].

Genome based resistance prediction and cluster analysis

Polymorphisms in 27 drug resistance associated genes that are involved in drug resistance mechanisms and three compensatory target genes (*rpoA*, *rpoC*, compensate fitness effects of *rpoB* mutations in RR strains[18] and *ahpC* upstream region, compensate fitness effects of catalase [*katG*] deficit in INH resistant strains[19]) were analyzed (Table S2).

Wild type gene sequences (*M. tuberculosis* H37Rv reference sequence or synonymous/silent mutations) were interpreted susceptible, known resistance variants [20] were considered as resistant, as well as insertion and deletions in the following genes: *katG, rpoB, pncA, ethA, Rv0678, ald* and *ddn*.

Primary cluster analysis was done using the cgMLST method as described previously [21]. SNP-based phylogenies were calculated as described previously[20]. Details are described in supplemental methods (Text S1).

Statistics

Statistical methods are detailed in the supplemental methods (Text S1).

Results

Study population

Over the study period, MTBC strains were obtained from 2 040 patients. These strains were sent to the laboratory of the tertiary care hospital in Mumbai from private physicians practicing in India, mainly the Mumbai Metropolitan Region (Figure S1).

WGS data quality was sufficient for 1 852 strains (90.8 %), while WGS data of 188 isolates were excluded from data analysis: 22 had a coverage of below 40x, in seven samples the proportion of unambiguous reads were below 85 %, another 40 samples had mixed infections with two MTBC strains and 119 had technical errors arising due to discrepancies in levofloxacin drug susceptibility testing between genotypic and phenotypic DST (Figure 1).

Of the 1 852 patients, 55.5 % (n = 1 027) were female, and 44.5 % (n = 824) were male. TB cases were mostly diagnosed within the age group of 18 to 40 years (n = 1 081; 58.4 %) and were widely distributed in Mumbai and its suburban region (Figure S1, Table S3). The mean age of the whole population was 33.7 years (SD 16.3). All data are summarized in Table S4.

Drug resistance and MTBC population structure

1 017 were classified as MDR (54.9 %) and 835 as not MDR, including 681 (36.8 %) pan-susceptible strains and 154 strains with variable resistances other than MDR (8.3 %, Figure 2, Table S3). Among the 1 017 MDR strains, 588 strains were pre-XDR (31.7 % of the total population, and 57.8 % of the MDR MTBC strains), and 182 XDR (9.8 % of the total population, and 17.9 % of the MDR MTBC strains, Figure 2, Table S3, Table S4).

Lineage 2 strains (L2, Beijing/East Asia) constituted 41 % of the total collection (n = 756), followed by Lineage 3 (L3, Delhi/CAS, n = 531, 29 %), Lineage 1 (L1, East Africa Indian, EAI, n = 303, 16 %) and Lineage 4 (L4, Euro-American, n = 260, 14 %) strains (Figure 2). L2 strains are overrepresented in drug resistance strains, especially in those harboring multiple drug resistances, while strains of the other

three lineages show an opposite trend. Indeed, 77 % of all pre-XDR and 86 % of all XDR strains belong to L2 (Table S3).

Genome based cluster analysis

A cgMLST-based cluster analysis employing a threshold of a maximum allele distance of 12 alleles grouped 801 (43 %) of the 1 852 strains into 96 clusters, ranging in size from two to 258 strains (Figure S2, Table S4). The three biggest clusters comprise 258 (cluster 1), 127 (cluster 2) and 87 (cluster 3) strains. Clustered strains comprise all four major MTBC-lineages, however, all strains of the three largest allele clusters were L2 strains (Figure 2, Table S3). If the different resistance categories were considered, the cluster rate was 55.1 % in MDR, 77.7 % in pre-XDR and 84.6 % in XDR strains (Table S3).

Cluster 1-3 strains were close to 100 % resistant to INH, RMP, EMB, PZA, and SM, while only PZA resistance rate was lower in cluster 3 strains (11.5 %, Figure S3) and also developed high FQ resistance rates (84.1 %) rendering them at least pre-XDR (see below). Resistance to injectable drugs was 42.2 % in cluster 1 strains, thus, 96 out of the 258 cluster 1 strains were classified as XDR (Figure 2, Table S3).

Factors associated with pre-XDR/XDR-TB

In the univariate statistical analysis, sex and age group were not associated with pre-XDR/XDR-TB (Table 1). L2 strains ($P < 0.001$), belonging to a cluster ($P < 0.001$), and belonging to clusters 1-3 increased the odds of pre-XDR/XDR compared to MDR, and L1, 3, and 4 strains had a lower odd of being pre-XDR/XDR compared to MDR (Table 1).

In multivariate analysis, the odds of a strain being pre-XDR/XDR was twice as high for strains belonging to L2 (aOR 2.07, 95% CI 1.24, 3.46) and for strains belonging to a cluster 2 (aOR 2.37, 95% CI 1.17, 4.83), 50% lower for strains belonging to lineage 3 (aOR 0.51, 95% CO 0.31, 0.85) and 80 % lower for strains belonging to lineage 1 (aOR 0.18, 95 % CI 0.31, 0.85; Table 1 and Table S5).

High resolution SNP-based analysis of cluster 1-3 MTBC strains

To get in-depth data on the transmission dynamics and evolution of the most dominant strains in our collection, we performed a high-resolution SNP-based analysis on the L2 strains of allele clusters 1-3. Using a L2 sub-classification system[8], cluster 1 strains were classified as Asian/African 2 subgroup, whereas cluster 2 strains were classified as Ancestral 3 strains. Strains from cluster 3 could not be assigned to a particular previously defined L2 subgroup (Table S4).

Using a maximum SNP distance of 12, a total of 239 cluster 1 strains could be grouped into eight SNP-based clusters (SNP_cl), ranging in size from two to 143 isolates (Figure 3, Table S4). The largest SNP cluster is SNP_cl 1 with 143 isolates, followed by SNP_cl 2 and SNP_cl 3, which comprise 37 and 41 isolates, respectively. The phylogeny of the strains in maximum likelihood phylogeny based on the concatenated SNP sequence (356 parsimony-informative, 961 singleton sites, 1 106 constant sites) is in line with particular resistance types, and, thus, confirmed the clonality of the isolates in particular sub-branches e.g. by carrying the same *rpoB*, *embB*, or *pncA* mutations (Figure 3).

The close relationship and likely transmission of pre-XDR and XDR strains was further confirmed by the grouping of isolates into several closely related subgroups in the phylogeny that share the same FQ and injectable drug resistance mutations e.g. *eis* -12c/t, *eis* -10g/t, *gyrA* 90V, or even double mutations such as *gyrA* 90V and *gyrA* D94G (Figure 3, Table S4), pointing towards a common ancestor that acquired these mutations before the clone started spreading as pre-XDR/XDR in the Mumbai area. In cluster 1 117 (89.3 %) of the 131 pre-XDR MTBC strains carried a mutation that leads to FQ resistance, which makes those strains already comparable with XDR MTBC strains from a clinical point of view. Only 13 (9.9 %) were resistant to an injectable drug, but susceptible to FQs.

To test if strains of particular subgroups were spreading in certain areas in Mumbai, we linked the SNP clusters with geographical occurrence (Figure 4). However, this analysis confirmed that strains of all cluster 1 SNP subgroups occur in all parts of the study region indicating wide-spread of these clones. Finally, we screened the NGS datasets for compensatory mutations that have been previously described to enhance the transmissibility of MDR strains[8,9,18]. This analysis revealed that 243 of the 258 cluster 1 strains carry at least one compensatory mutation in *rpoC* (Table S4).

SNP-based analysis of strains from cluster 2 and 3 confirmed their high clonality with virtually all strains belonging to 1 SNP group (Figure S4). The phylogeny of cluster 2 and 3 strains reveals a similar pattern than observed for cluster 1 strains. Sub-groups share same patterns of resistance mutations indicating their acquisition by a common ancestor followed by clonal transmission (Figure S4). This also applies for pre-XDR and/or XDR MTBC strains.

Discussion

Our large-scale genome-based study analyzing 1 852 MTBC strains from India, mainly the Mumbai Metropolitan region, revealed several remarkable findings shedding light on the MDR, pre-XDR and XDR epidemic in one of the highest populated metropolitan areas of the world. First, we determined high rates of pre-XDR, and XDR strains among the MDR strain population in this study. Indeed, 58.4 % of all MDR strains are already pre-XDR and 17.9 % are XDR. This remarkable shift towards pre-XDR is mediated by high frequencies of FQ resistance mutations that, in combination with particular injectable drug resistance mutations, result in XDR-TB. In addition, pre-XDR, and XDR strains are virtually all L2 strains and show high cluster rates as an indication of ongoing transmission.

On a global level, the WHO reports that approximately 6 % of the MDR-TB patients have an XDR-TB[2]. With 17.9 %, the XDR-TB rate is clearly higher in our study population. Additionally, we observed a high rate (58.4 %) of pre-XDR strains with FQ resistance, thus, rendering one of the most effective drugs of the short and long MDR-TB regimen non-effective[22]. Indeed, *gyrA* D94G (41%) and *gyrA* A90V (21%) were observed as the predominant mutations in the FQ resistant strains from India in the CRyPTIC project (unpublished data), the former mutation contributing to high level resistance (unpublished data).

To the best of our knowledge, a comparable shift of resistance from MDR towards pre-XDR and XDR has not been reported from other regions, though it has been evident in Mumbai over the last two decades[7,23,24]. Our data underline the potential of MDR MTBC strains to evolve further resistance and yet efficiently transmit even when they become pre-XDR and XDR.

Ongoing and effective transmission of MDR, pre-XDR, and XDR MTBC strains is further confirmed by subgroups of clustered strains, that share identical variant sites conferring resistance including FQ resistance and injectable drug resistance mutations. The clonal expansion of particular pre-XDR/XDR clones with combined resistance to all first line drugs and FQs reduces available group A, B, and C drugs proposed for the treatment of MDR-TB cases to a minimal set. This renders use of the short MDR-TB

regimen impossible for patients infected with such strains[2,22]. Geographical mapping showed that strains of the dominant cluster 1 are dispersed across all districts of the Mumbai metropolitan area of more than 28 million inhabitants.

A further striking finding of our study is strong association of L2 strains with pre-XDR/XDR-TB linked with a very high cluster rate (85 %) of L2 strains. Indeed, strains of the three major L2 clusters account for half of the pre-XDR and two thirds of all XDR-TB cases, and just cluster 1 strains account for 52.1 % of the XDR MTBC strains. The proportion of Beijing strains in Mumbai increased over the last two decades[7,23,25]. These data highlight the insights sequence based molecular epidemiological analysis reveals on how an MDR/pre-XDR/XDR epidemic emerges and becomes prevalent in a densely populated geographic area. In fact, the challenge to control pre-XDR/XDR-TB in Mumbai specifically requires intensification of drug resistance detection, effective drug treatment and transmission control of clusters 1-3 strain. Robust monitoring is required for detection and control of new emergent, fit, and transmissible multi-resistant lineages. An earlier study has already highlighted the competitive fitness of MDR strains from India using growth competition experiments [26]. An association between BCG vaccine escape and efficient spread of Beijing strains has also been proposed[27,28].

The clonality of the MDR/pre-XDR/XDR MTBC strains in our collection is remarkable. Previously, we determined dominant MDR clones in Eastern European settings, and could show that the spread of a few dominant MDR MTBC strains with compensatory mutations e.g. in *rpoC* can significantly affect the MDR-TB epidemic in specific regions[8,9,29,30]. However, pre-XDR and XDR-TB cases have until now been found at a comparably lower frequency and FQ resistance has not been present in more than 30% of the isolates [8,9,29,30]. Thus, our data extend these findings to pre-XDR/XDR-TB and demonstrate that pre-XDR/XDR-TB can be propelled by a few lineages that have obviously acquired compensatory mutations, transmit efficiently and reach high prevalence in the population.

Our study has limitations. We only investigated MTBC strains from the laboratory of one tertiary care hospital in Mumbai which represents community samples from a pool of private physicians from the MMR. Although our study covered approx. 16 % of MDR cases in occurring in the region, it may not be

representative for the whole Mumbai metropolitan area and for the rest of India. Still, our geographical mapping shows that the study captured cases from the main districts of Mumbai as well as from other areas in India. However, considering our findings, larger investigations on the MDR/pre-XDR/XDR proportions and on the spread of dominant pre-XDR/XDR MTBC strains in Mumbai and other parts of India urgently need to be performed.

In conclusion, our data indicate that the majority of pre-XDR/XDR-TB cases are caused by highly transmissible L2 lineages. Thus, successful control in Mumbai of the DR epidemic urgently requires measures for stopping the transmission of MDR/pre-XDR/XDR strains of the Beijing lineage. As the majority of pre-XDR strains are already FQ resistant, treatment options are limited and rapid adaptation of treatment strategies, for example, comprehensive resistance detection for better design of personalized effective treatment regimens need to be established. It is likely, that the uninformed use of treatment regimens including the newest MDR-TB drugs without precise knowledge of individual resistance patterns and close patient monitoring will likely result in further resistance development as described already[31–34] and ongoing transmission of even more resistant strains. The national extent of spread in India by the dominant pre-XDR/XDR clones identified in Mumbai and other regions needs to be urgently considered.

Acknowledgments

Parts of this work have been supported within the CRYPTIC consortium by the Bill & Melinda Gates Foundation [OPP1133541] and the Wellcome Trust [200205/Z/15/Z], the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germanys Excellence Strategy – EXC 2167 Precision Medicine in Inflammation, and the Leibniz Science Campus Evolutionary Medicine of the LUNG (EvoLUNG). DWC is funded by the UK National Institutes of Health Research (NIHR) Biomedical Research Centre, Oxford. The funders had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Author contributions

VD, AM, PD, MM, KK, IB, CU, DWC, NM, and SN conceived the idea and designed the study, and analysed and interpreted the data. All authors contributed to obtaining and assembling the data. VD, AM, and SN wrote the initial draft of the paper. All authors contributed to data interpretation, final draft of the paper and approved the final version of the manuscript.

References

1 WHO | Global tuberculosis report 2020. WHO. http://www.who.int/tb/publications/global_report/en/ (accessed Nov 4, 2020).

2 WORLD HEALTH ORGANIZATION. GLOBAL TUBERCULOSIS REPORT 2019. S.l.: WORLD HEALTH ORGANIZATION, 2019.

3 Dheda K, Gumbo T, Maartens G, *et al.* The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. *The Lancet Respiratory Medicine* 2017; **5**: 291–360.

4 Mathema B, Kurepina NE, Bifani PJ, Kreiswirth BN. Molecular Epidemiology of Tuberculosis: Current Insights. *Clin Microbiol Rev* 2006; **19**: 658–85.

5 Yates TA, Khan PY, Knight GM, *et al.* The transmission of Mycobacterium tuberculosis in high burden settings. *The Lancet Infectious Diseases* 2016; **16**: 227–38.

6 Sharma P, Verma M, Bhilwar M, *et al.* Epidemiological profile of tuberculosis patients in Delhi, India: A retrospective data analysis from the directly observed treatment short-course (DOTS) center. *Journal of Family Medicine and Primary Care* 2019; **8**: 3388.

7 Dalal A, Pawaskar A, Das M, *et al.* Resistance Patterns among Multidrug-Resistant Tuberculosis Patients in Greater Metropolitan Mumbai: Trends over Time. *PLoS One* 2015; **10**. DOI:10.1371/journal.pone.0116798.

8 Merker M, Blin C, Mona S, *et al.* Evolutionary history and global spread of the Mycobacterium tuberculosis Beijing lineage. *Nat Genet* 2015; **47**: 242–9.

9 Merker M, Barbier M, Cox H, *et al.* Compensatory evolution drives multidrug-resistant tuberculosis in Central Asia. *Elife* 2018; **7**. DOI:10.7554/eLife.38200.

10 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**: 1181–2.

11 Manson AL, Abeel T, Galagan JE, *et al.* Mycobacterium tuberculosis Whole Genome Sequences From Southern India Suggest Novel Resistance Mechanisms and the Need for Region-Specific Diagnostics. *Clin Infect Dis* 2017; **64**: 1494–501.

12 Chatterjee A, Nilgiriwala K, Saranath D, Rodrigues C, Mistry N. Whole genome sequencing of clinical strains of Mycobacterium tuberculosis from Mumbai, India: A potential tool for determining drug-resistance and strain lineage. *Tuberculosis (Edinb)* 2017; **107**: 63–72.

13 Advani J, Verma R, Chatterjee O, *et al.* Whole Genome Sequencing of Mycobacterium tuberculosis Clinical Isolates From India Reveals Genetic Heterogeneity and Region-Specific Variations That Might Affect Drug Susceptibility. *Front Microbiol* 2019; **10**: 309.

14 Sethi S, Hao Y, Brown SM, *et al.* Elucidation of drug resistance mutations in Mycobacterium tuberculosis isolates from North India by whole-genome sequencing. *J Glob Antimicrob Resist* 2020; **20**: 11–5.

15 The Times of India. Drug-resistant TB cases in Mumbai up 36% in 3 years. Read more at: http://timesofindia.indiatimes.com/articleshow/63436898.cms?utm_source=contentofinterest&utm_medium=text&utm_campaign=cppst (accessed Jan 20, 2021).

16 Kohl TA, Utpatel C, Schleusener V, *et al.* MTBseq: a comprehensive pipeline for whole genome sequence analysis of Mycobacterium tuberculosis complex isolates. *PeerJ* 2018; **6**: e5895.

17 Coll F, McNerney R, Guerra-Assunção JA, *et al.* A robust SNP barcode for typing Mycobacterium tuberculosis complex strains. *Nat Commun* 2014; **5**. DOI:10.1038/ncomms5812.

18 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* 2012; **44**: 106–10.

19 Sherman DR, Mdluli K, Hickey MJ, *et al.* Compensatory ahpC gene expression in isoniazid-resistant Mycobacterium tuberculosis. *Science* 1996; **272**: 1641–3.

20 Merker M, Kohl TA, Barilar I, *et al.* Phylogenetically informative mutations in genes implicated in antibiotic resistance in Mycobacterium tuberculosis complex. *Genome Medicine* 2020; **12**: 27.

21 Kohl TA, Harmsen D, Rothgänger J, Walker T, Diel R, Niemann S. Harmonized Genome Wide Typing of Tubercle Bacilli Using a Web-Based Gene-By-Gene Nomenclature System. *EBioMedicine* 2018; **34**: 131–8.

22 Dheda K, Gumbo T, Maartens G, *et al.* The Lancet Respiratory Medicine Commission: 2019 update: epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant and incurable tuberculosis. *Lancet Respir Med* 2019; **7**: 820–6.

23 D'souza DT, Mistry NF, Vira TS, *et al.* High levels of multidrug resistant tuberculosis in new and treatment-failure patients from the Revised National Tuberculosis Control Programme in an urban metropolis (Mumbai) in Western India. *BMC Public Health* 2009; **9**. DOI:10.1186/1471-2458-9-211.

24 Kambli P, Ajbani K, Kazi M, *et al.* Targeted next generation sequencing directly from sputum for comprehensive genetic information on drug resistant Mycobacterium tuberculosis. *Tuberculosis (Edinb)* 2021; **127**: 102051.

25 Almeida D, Rodrigues C, Ashavaid TF, Lalvani A, Udwadia ZF, Mehta A. High incidence of the Beijing genotype among multidrug-resistant isolates of Mycobacterium tuberculosis in a tertiary care center in Mumbai, India. *Clin Infect Dis* 2005; **40**: 881–6.

26 Bhatter P, Chatterjee A, D'souza D, Tolani M, Mistry N. Estimating Fitness by Competition Assays between Drug Susceptible and Resistant Mycobacterium tuberculosis of Predominant Lineages in Mumbai, India. *PLOS ONE* 2012; **7**: e33507.

27 Kremer K, van der Werf MJ, Au BKY, *et al.* Vaccine-induced Immunity Circumvented by Typical *Mycobacterium tuberculosis* Beijing Strains. *Emerg Infect Dis* 2009; **15**: 335–9.

28 Hanekom M, van der Spuy GD, Streicher E, *et al.* A Recently Evolved Sublineage of the Mycobacterium tuberculosis Beijing Strain Family Is Associated with an Increased Ability to Spread and Cause Disease. *Journal of Clinical Microbiology* 2007; **45**: 1483–90.

29 Merker M, Nikolaevskaya E, Kohl TA, *et al.* Multidrug- and Extensively Drug-Resistant Mycobacterium tuberculosis Beijing Clades, Ukraine, 2015. *Emerging Infect Dis* 2020; **26**: 481–90.

30 Engström A, Antonenka U, Kadyrov A, *et al.* Population structure of drug-resistant Mycobacterium tuberculosis in Central Asia. *BMC Infect Dis* 2019; **19**: 908.

31 Andres S, Merker M, Heyckendorf J, *et al.* Bedaquiline-Resistant Tuberculosis: Dark Clouds on the Horizon. *Am J Respir Crit Care Med* 2020; **201**: 1564–8.

32 Kranzer K, Kalsdorf B, Heyckendorf J, *et al.* New World Health Organization Treatment Recommendations for Multidrug-Resistant Tuberculosis: Are We Well Enough Prepared? *Am J Respir Crit Care Med* 2019; **200**: 514–5.

33 Polsfuss S, Hofmann-Thiel S, Merker M, *et al.* Emergence of Low-level Delamanid and Bedaquiline Resistance During Extremely Drug-resistant Tuberculosis Treatment. *Clin Infect Dis* 2019; **69**: 1229–31.

34 Hoffmann H, Kohl TA, Hofmann-Thiel S, *et al.* Delamanid and Bedaquiline Resistance in Mycobacterium tuberculosis Ancestral Beijing Genotype Causing Extensively Drug-Resistant Tuberculosis in a Tibetan Refugee. *Am J Respir Crit Care Med* 2016; **193**: 337–40.

Table 1. Characteristics of MDR and pre-XDR/XDR cases with factors associated with pre-XDR/XDR-TB based on univariate and multivariate analysis.

	MDR		**pre-XDR/XDR**		**Univariate analysis**				**Multvariate logistic regression**			
	n	%	n	%	OR	95% lower	95% upper	P-value	Adjusted OR	95% lower	95% upper	Adjusted P-value
Total	247	100%	770	100%								
Gender												
M	101	41%	333	43%	1.09	0.82	1.46	0.55				
F	145	59%	437	57%								
unknown	1	0%	0	0%								
Age												
<18	34	14%	96	12%	0.89	0.58	1.40	0.59				
18-40	155	63%	502	65%	1.11	0.82	1.51	0.49				
40-60	41	17%	143	19%	1.15	0.77	1.72	0.51				
>60	16	6%	29	4%	0.57	0.29	1.14	0.08				
unknown	1	0%	0	0%								
Lineage												
Lineage 1 (EAI)	37	15%	13	2%	0.10	0.05	0.19	< 0.001	0.18	0.08525	0.378876	< 0.001
Lineage 2 (Beijing)	103	42%	611	79%	5.36	3.90	7.39	< 0.001	2.07	1.24238	3.461374	0.01
Lineage 3 (Delhi-CAS)	69	28%	70	9%	0.26	0.18	0.38	< 0.001	0.51	0.30597	0.853136	0.01
Lineage 4 (Euro-American)	38	15%	76	10%	0.60	0.39	0.94	0.02				
M. bovis	0	0%	0	0%								
Clustering												
Yes	136	55%	611	79%	3.13	2.28	4.30	< 0.001	0.89	0.59700	1.31551	0.55
No	111	45%	159	21%								
Cluster												
Cluster 1	31	13%	227	29%	2.91	1.92	4.53	< 0.001	1.65	0.98703	2.754255	0.06
Cluster 2	11	4%	116	15%	3.80	2.00	7.97	0.003	2.37	1.16704	4.831159	0.02
Cluster 3	15	6%	72	9%	1.59	0.88	3.06	0.12	1.08	0.56019	2.085229	0.82

Figures

Figure 1. Study flowchart. In- and exclusion criteria for strains are reported in the two rhombuses. Final dataset consists of 1 852 strains.

Figure 2. Genotype and Resistance category distribution of strains across 1 852 isolates (Total) and within the three major clusters.

A. Distribution of genotypes across the 1 852 isolates; Lineage 1 (EAI and EAI Manila), Lineage 2 (Beijing), Lineage 3 (Delhi- CAS) and Lineage 4 (Euro- American, H37Rv-like, Haarlem, LAM, mainly T, S-type, Ural and X-type); For the total dataset the main lineages are Lineage 2 (41 %), followed by Lineage 3 (29 %) and Lineage 1 (16 %). All strains in cluster 1,2 and 3 belong to Lineage 2. **B.** Distribution of resistance category across the 1852 isolates; Resistance categories are RR (Rifampicin Resistant), non MDR (resistant, but not multi drug resistant [MDR]), MDR, pre-XDR (pre-extensively drug resistant) and XDR (extensively drug resistant); of the total strain population about 37 % are susceptible (S) to all drugs, 32 % of strains are pre-XDR whereas 10 % are XDR in the cohort. The resistant category distribution of cluster 1, 2 and 3 differs, with all strains being at least MDR and up to 37 % of strains being already XDR (Cluster 1).

Figure 3. Maximum likelihood phylogeny based on the concatenated SNP sequence of 258 MTBC strains from allele-based cluster 1. The concatenated SNP sequence consists of 356 parsimony-informative, 961 singleton sites and 1 106 constant sites; mutations related to respective drugs and resistance status are color coded and expressed as annotation rings on the tree. SNP-based clusters with maximum distance of 12 (d12) is plotted on the outer ring. Abbreviations: INH, Isoniazid; EMB, Ethambutol; PZA, Pyrazinamide; FQ, Fluoroquinolones.

Figure 4: Geographical occurrence of patients with maximum SNPs distance of 12 for Cluster 1 across Mumbai Metropolitan Region (MMR). The geographical distribution underlines the widespread of all cluster 1 SNP subgroups across the city and its neighboring areas. Boundaries of the map for the neighboring regions are not available online.

Supplemental data

Table S1. ENA accession numbers.

Table S2. Genes analyzed.

Table S3. Characteristics of analyzed samples divided by resistance group.

Table S4. Data table

Table S5. Multivariate logistic regression results

Figure S1. Geographical distribution of the strains investigated. Strains were plotted on a map according to the geographical position of the submitting center and color coded by the resistance category. All categories came from the whole study region.

Abbreviations: S- Susceptible to all drugs, nonMDR – resistant but not multi-drug resistant, MDR – multi-drug resistant, pre-XDR – pre-extensively drug resistant, XDR – extensively drug resistant

Figure S2. Minimum spanning tree based on the analysis of 2 891 alleles of the core genome of the 1 852 *M. tuberculosis* complex strains investigated. Missing values were ignored for pairwise comparisons. Strains are color-coded by the respective lineage name. EAI and EAI Manila (Lineage 1), Beijing (Lineage 2), Delhi- CAS (Lineage 3) and Euro- American, H37Rv-like, Haarlem, LAM, mainly T, S-type, Ural and X-type (Lineage 4). Allele clusters are highlighted by color-shaded branches.

Figure S3. Barplot of resistance profiles of the strains belonging to allele cluster 1, 2 and 3. Resistance profiles in %. A) Resistance profile of allele Cluster 1, B. Resistance profile of allele Cluster 2, C. Resistance profile of allele Cluster 3

Abbreviations: INH - Isoniazid; RMP - Rifampicin; SM - Streptomycin; EMB - Ethambutol; PZA - Pyrazinamide; MFX - Moxifloxacin; LFX - Levofloxacin; CFZ - Clofazimine; KAN - Kanamycin; AMI - Amikacin; CPR - Capreomycin; ETH - Ethionamide; LZD - Linezolid; BDQ - Bedaquiline; CS - Cycloserine; PAS - para-aminosalycilic acid; DEL - Delamanid

Figure S4. Maximum likelihood (ML) phylogeny of strains belonging to cluster 2 and 3. Mutations related to respective drugs and resistance status are color coded on the annotation rings of the tree. A. ML phylogenetic tree of the 127 MTBC strains from Cluster 2 phylogeny is based on the concatenated SNP sequence with 1 110 parsimony-informative and 386 singleton sites B. ML phylogenetic tree of the 87 MTBC strains from Cluster 3, phylogeny is based on the concatenated SNP sequence with 122 parsimony-informative and 395 singleton sites Abbreviations: INH - Isoniazid; EMB - Ethambutol; PZA - Pyrazinamide; FQ - Fluoroquinolones.

Figure 1

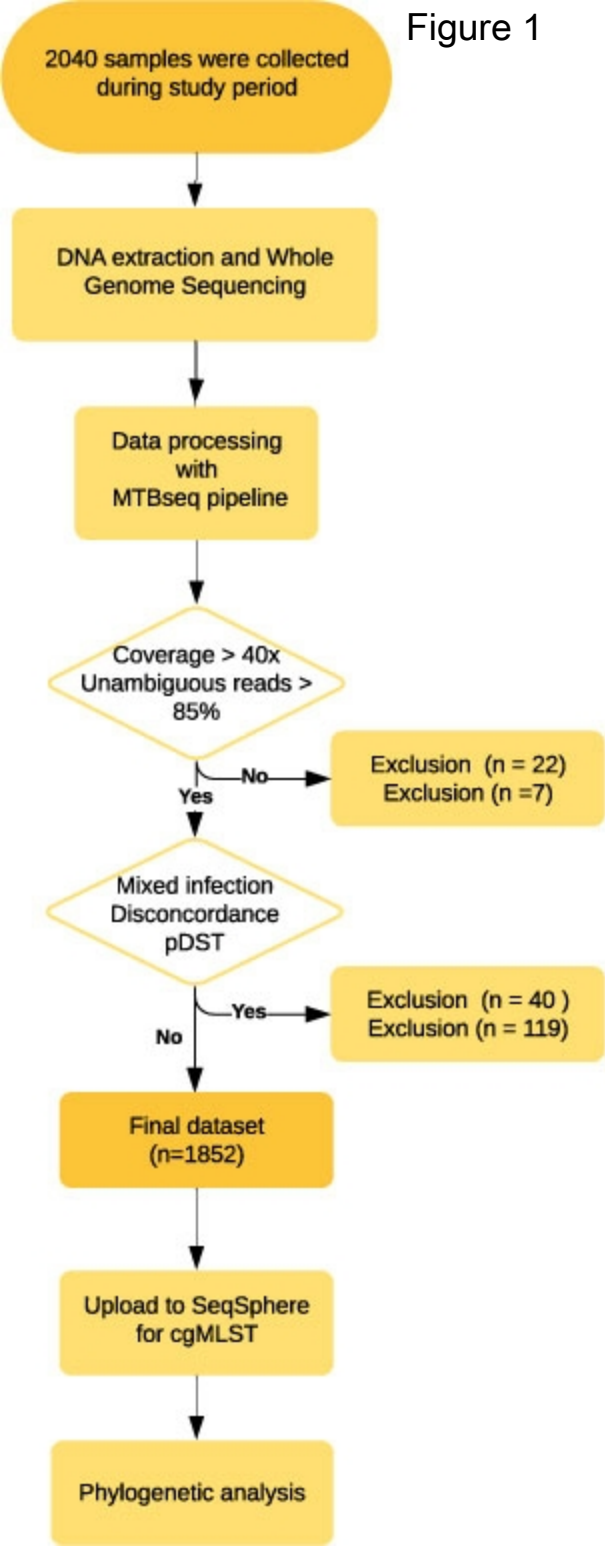

2040 samples were collected during study period
DNA extraction and Whole Genome Sequencing
Data processing with MTBseq pipeline
Coverage > 40x Unambiguous reads > 85%
No
Exclusion (n = 22)
Exclusion (n =7)
Yes
Mixed infection Disconcordance pDST
Yes
Exclusion (n = 40)
Exclusion (n = 119)
No
Final dataset (n=1852)
Upload to SeqSphere for cgMLST
Phylogenetic analysis

Figure 2 A

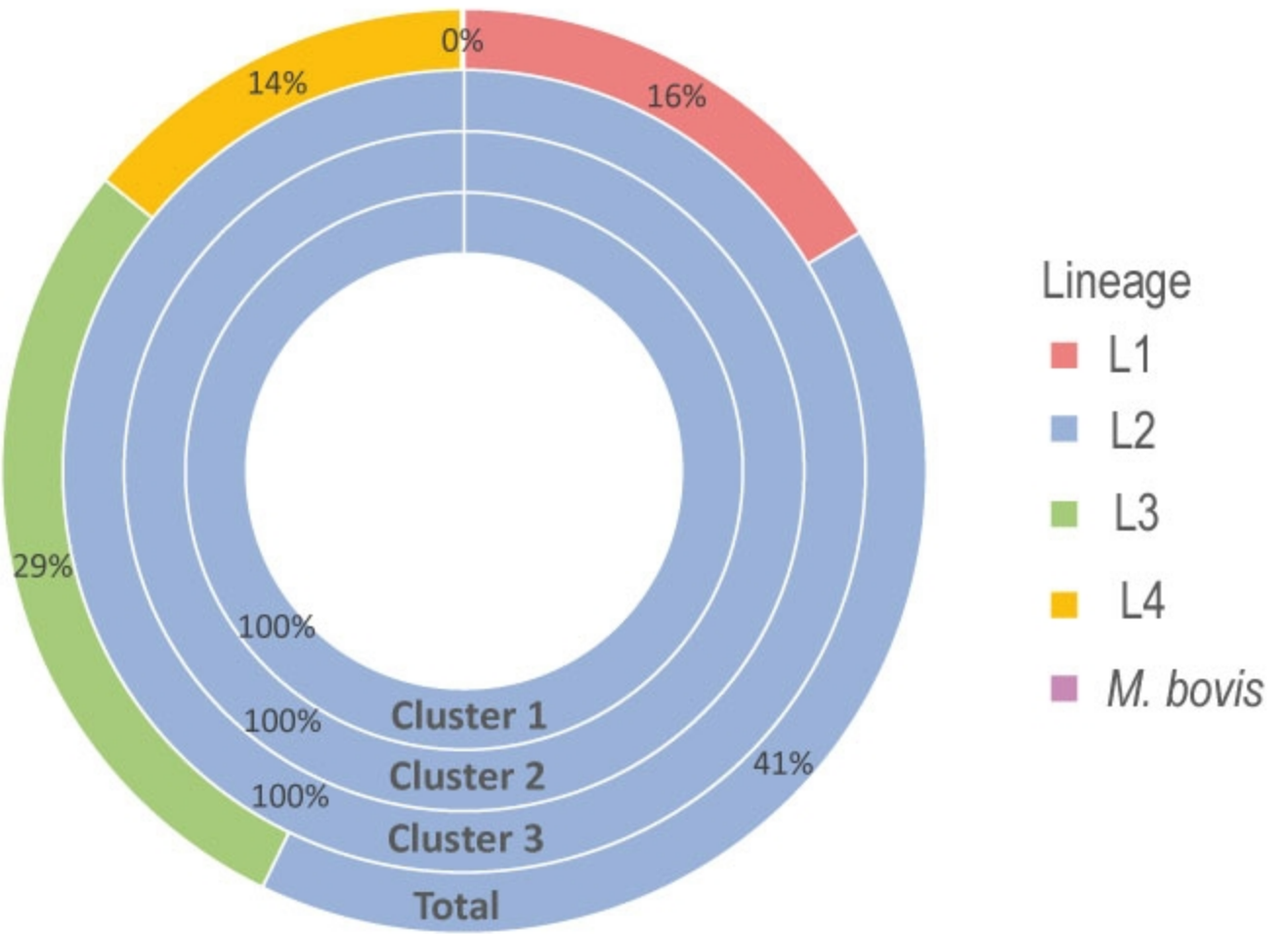

0%
16%
14%
29%
41%
100%
100%
100%
Cluster 1
Cluster 2
Cluster 3
Total
Lineage
L1
L2
L3
L4
M. bovis

Figure 2 B

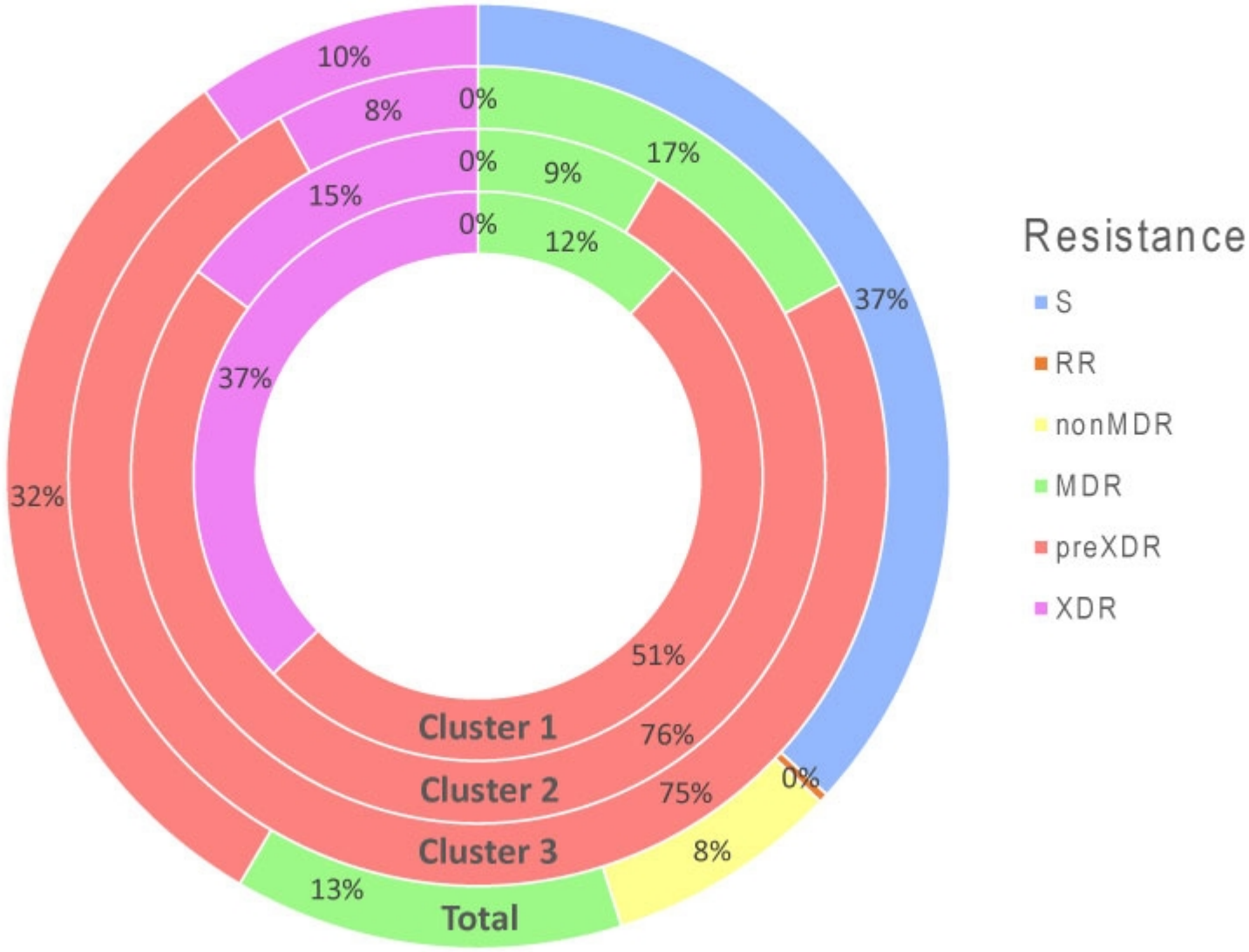
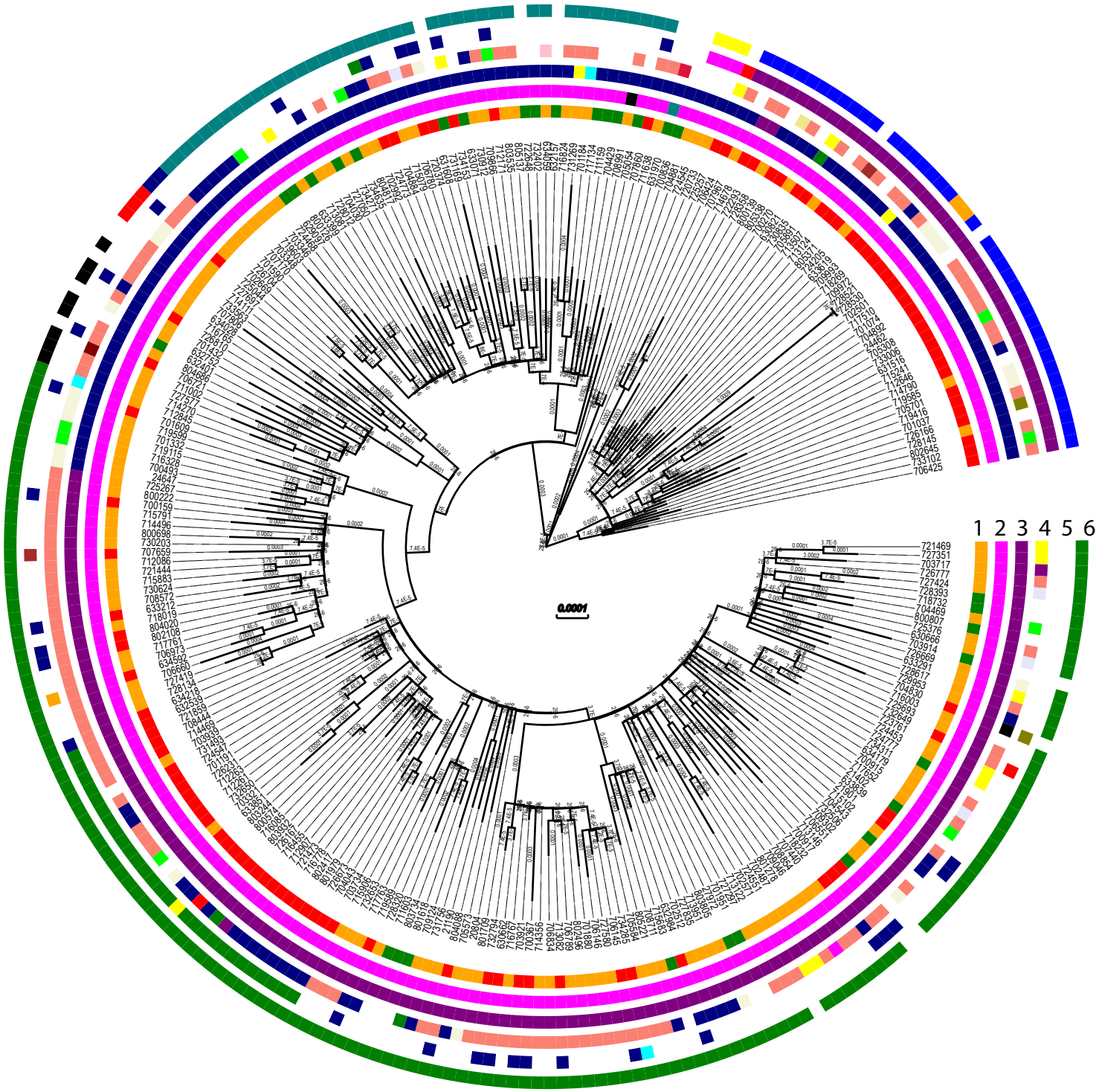

10%
8%
0%
0%
15%
9%
17%
0%
12%
37%
37%
32%
51%
Cluster 1
76%
Cluster 2
75%
0%
Cluster 3
8%
13%
Total
Resistance
S
RR
nonMDR
MDR
preXDR
XDR

Figure 3



1 Resistance status
- MDR
- preXDR
- XDR

2 rpoB mutation
- rpoB Q432K
- rpoB S450L

3 INH mutations
- katG S315T
- fabG1 -15c>t and katG S315T
- fabG1 -8t>a and katG S315T
- fabG1 -8t>c and katG S315T
- fabG1 L203L and katG S315T
- fabG1 L203L and katG S315T

4 FQ mutations
- gyrA A90V
- gyrA A90V and gyrA D94A
- gyrA A90V and gyrA D94G
- gyrA A90V and gyrA D94N
- gyrA A90V and gyrA S91P
- gyrA A90V and gyrA S91P and gyrA D94G
- gyrA D94A
- gyrA D94G
- gyrA D94H
- gyrA D94N
- gyrA D94Y
- gyrA S91P
- gyrB D461A
- gyrB D461N
- gyrB N499T
- gyrB N499T and gyrA D94A
- gyrB N499T and gyrA D94A
- gyrB T500P

5 Injectable mutations
- rrs 1401a>g
- eis -12c>t
- eis -10g>c
- eis -37g>t
- rrs 1484g>t
- eis -10g>a
- rrs 1401a>g and eis -10g>c
- rrs 1401a>g and eis -12c>t
- eis -12c>t and eis -37g>t
- rrs 1401a>g and eis -14c>t

6 SNP cluster d12
- SNP_cl 1
- SNP_cl 2
- SNP_cl 3
- SNP_cl 4
- SNP_cl 5
- SNP_cl 6
- SNP_cl 7
- SNP_cl 8

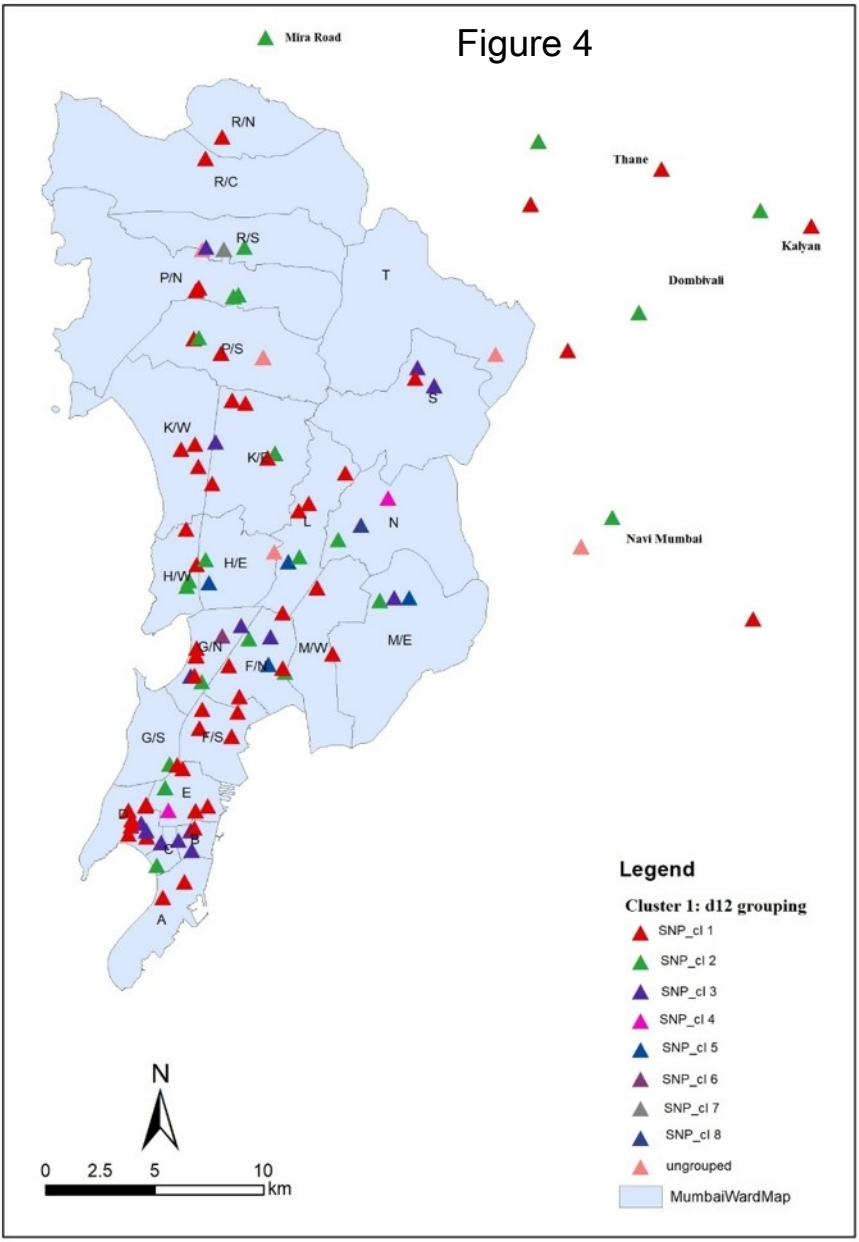


Figure 4
Mira Road
Thane
Kalyan
Dombivali
Navi Mumbai
R/N
R/C
R/S
P/N
P/S
T
S
K/W
K/E
L
N
H/E
H/W
G/N
F/N
M/W
M/E
G/S
F/S
E
A
N
0 2.5 5 10
km
Legend
Cluster 1: d12 grouping
SNP_cl 1
SNP_cl 2
SNP_cl 3
SNP_cl 4
SNP_cl 5
SNP_cl 6
SNP_cl 7
SNP_cl 8
ungrouped
MumbaiWardMap