Characterisation of drug-resistant *Mycobacterium tuberculosis* mutations and transmission in Pakistan

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## ABSTRACT

GARY TO WRITE

## INTRODUCTION

Tuberculosis disease (TB), caused by bacteria in the Mycobacterium tuberculosis (*Mtb*) complex, is a major global public health problem. Pakistan is a high-burden TB country, being one of eight countries accounting for two-thirds of the estimated 10 million people globally that fell ill with the disease 1. In 2019, Pakistan had a total TB incidence of 570,000 and 43,900 deaths 1, but disease control is being compromised by increasing HIV prevalence and drug resistance. The country has a high burden for rifampacin resistant (RR-TB) and multidrug-resistance (MDR-TB), which is the additional resistance to isoniazid treatments. There were 25,000 cases of MDR-/RR-TB in 2019 1. The National TB control program aims to reduce by half the prevalence of TB in the general population by 2025, but to achieve this will require the scaling-up of TB detection and clinical care, as well as improved systems for inferring disease transmission, thereby facilitating further targeted interventions.

Whole genome sequencing (WGS) is revolutionizing our understanding of drug resistance and clinical management, as well as transmission patterns, thereby assisting disease control 2. *M. tuberculosis* drug resistance is linked to genomic variants in drug targets or pro-drug activators, including single nucleotide polymorphisms (SNPs) and small insertions and deletions (indels), some occurring in gene-gene interactions. It is therefore possible to predict resistance genotypically for 19 anti-TB drugs and their groups (e.g. floroquinolines) using curated libraries of >1000 mutations across >30 loci 3,4, thereby personalizing treatment. Genotypic predictions are an alternative to bacterial culture-based phenotypic drug susceptibility testing (DST), which can be time-consuming and resource intensive, with reproducibility and inhibitory concentration cut-off challenges for particular drugs 3. Further, WGS data infers the population structure with the *M. tuberculosis* complex, which is phylo-geographical in nature, with strains falling within distinct (sub-)lineages 5, and potential transmission chains identified through isolates with (near-)identical genomic variation 6. The identification of highly virulent strain-types or lineages, drug resistance, and transmission clusters will assist the targeting of limited resources for TB control.

There have been recent studies using WGS to characterize *M. tuberculosis* genetic diversity in isolates sourced from Pakistan, where the predominant strains are from the Central Asian (CAS) family, set within lineage 3 7–12. A recent study of TB endemic province of Khyber Pakhtunkhwa (North West Pakistan) found that known mutations in *rpoB* (e.g. S405L), *katG* (e.g. S315T), or *inhA* promoter loci explain the majority of MDR-TB, but there was evidence of complex mixed infections and heteroresistance, which may reflect the high transmission nature of the setting 12. An earlier study in the same province found similar MDR-TB mutations, but also others in genes conferring resistance to other first and second-line drugs, including in *pncA* (pyrazinamide), *embB* (ethambutol), *gyrA* (fluoroquinolones), *rrs* (aminoglycosides), *rpsL, rrs* and *giB* (streptomycin) loci. Further, acquisition of rifampicin resistance often preceded isoniazid in our isolates, but worringly a high proportion (~18%) of pre-MDR isolates had fluoroquinolone resistance markers, potentially due to unregulated anti-TB drug use 7. Eighteen *M. tuberculosis* isolates clustered within eight networks, thereby providing evidence of drug-resistant TB transmission in the Khyber Pakhtunkhwa province 7. An investigation of XDR-TB isolates sourced across four provinces found similar genes linked to drug resistance as in Khyber Pakhtunkhwa 10, and follow-up work found an increased frequency and expression of novel SNP mutations in efflux pump genes, potentially explaining some drug resistance mechanisms10.

Here, we analyse 535 *M. tuberculosis* samples with WGS data , collected between 2003 and 2020, with phenotypic testing of resistance across 12 drugs (rifampicin, isoniazid, ethambutol, pyrazinamide, streptomycin, ofloxacin, moxifloxacin, amikacin, kanamycin, capreomycin, ciprofloxacin, ethionamide). By identifying ~38k SNPs, and inferring genotypic drug resistance across 19 anti-TB drugs (as well as fluoroquinolones and aminoglycosides classes), we sought to understand the phylogeny of *M. tuberculosis* in the largest Pakistan dataset, identify transmission events, and infer commonly circulating mutations linked to drug resistance. The genetic insights were validated in a large *M. tuberculosis* collection (n=34k) with WGS and drug susceptibility test data5.

**RESULTS**

### Isolates and whole genome sequencing data

A total of 535 *M. tuberculosis* isolates sourced between 2003 and 2005, and 2013 and 2020 from Pakistan with publically available WGS and phenotypic susceptibility testing 7–12. These isolates covered at least four provinces (Balochistan, Khyber Pakhtunkhwa, Punjab, Sindh), but a high proportion of locations were missing (69.5%), all from one study 11 (**Table 1**). The majority of samples were from lineage 3 (L3 397, 74.2%; CAS strains), but the other main lineages were represented (L4, 80, 15.0%, including LAM, T and X strains); L2 36, 6.7%, including Beijing; L1 22, 4.1%) (**Table 1; S1 Table).**

Phenotypic drug susceptibility testing (DST) was most complete for rifampicin (n=487, 91.03%), isoniazid (n=487, 91.03%), ethambutol (n=479, 89.53%), and pyrazinamide (n=444, 82.99%) drugs **(S2 Table**), but incomplete / not present for 12 drugs.A total of 432 total samples (80.7%) were phenotypically resistant to at least one drug (median 3, maximum 10). The number of potential errors on the phenotypic testing appeared modest (218/2,430 tests, 9.0%), where established genotypic resistance markers were present in isolates with DST results that implied drug susceptibilty. The discordance appeared in nine drugs (capreomycin, ethambutol, ethionamide, isoniazid, moxifloxacin, ofloxacin, pyrazinamide, rifampicin, streptomycin), but predominantly in ethambutol (96) (**S2 Table**). The majority of isolates were genotypically assessed as MDR-TB (328, 61.3%), with high proportions of (pre-) XDR (113, 21.1) and pan-sensitive (60, 11.2%) (**Table 1**). There were 31 pre-MDR, and overall there was a high prevalence of rifampicin (460, 86.0%) and isoniazid (435, 81.3%) resistance strains. Resistance to drugs used in combination with isoniazid and rifampicin were also common, including ethambutol (385, 72.0%), pyrazinamide (258, 48.2%), streptomycin (238, 44.5%), ethionamide (102, 19.1%), any fluoroquinolones (277, 51.8%) or aminoglycoside (75, 14.0%). As expected, very few isolates appeared resistant to bedaquiliine, clofazimine and cycloserine (n<3; **Table 1**). Across all lineages, the majority of isolates (>75%) were at least MDR-TB resistance (**S3 Table**).

After sequence data alignment, high average coverage was observed across the samples (median 76-fold, range 30-2,027). A total of 37,970 SNPs were identified genome-wide, with 23,741 (62.5%) found in single isolates. A phylogenetic tree constructed using the 37,970 genome-wide SNPs has the expected lineage-based clustering (**Figure 1**). The tree also revealed clades, including pre-XDR and XDR samples, which are highly similar and potential outbreaks or transmission related.

**Evidence of transmission**

The median (range) pairwise SNP differences across the 535 isolates was 390 (minimum 0, maximum 1811), with a multi-modal distribution, where modes representing differences within and between lineages (**S2 Figure**). At a threshold of 10 SNPs, 55 clusters formed consisting of a total of 169 isolates, where the median number of isolates in each cluster was 2 (range: 2 - 22) (**S2 Figure**). The effects of changing the SNP threshold on clusters number and sizes (**S4 Table**) indicated the presence of only six additional clusters (33 more isolates) when increasing from a stringent threshold (5 SNPs) to our pragmatic choice (10 SNPs).

“Transmitted” isolates were found in three of the four provinces recorded (Khyber Pakhtunkhwa 71/169; Punjab 9/169; Sindh 9/169), identified across all lineages Lineage 1: 7/169, Lineage2: 21/169, Lineage3: 98/169, Lineage4: 43/169) and in (pre-)XDR (75/169) samples (**S3 Figure; S4 Figure**).

Most clusters had samples with the same drug resistance phenotype ~~(49/55)~~ (44/55), and there was some evidence of clusters consisting of more than one location ~~(XXX/55)~~ (35/55, excluding missing locations) (**S3 Figure; S4 Figure**).

Comparing the 169 "transmitted" isolates in clusters to the others ("non-transmitted"; n=366), there were differences in lineage (Chi-sq P = 1.9x10-8) and drug resistance (Chi-sq P=0.0005). In particular, there was an increased risk of transmission in lineages 2 (OR etc. ) and 4 (OR etc.), compared to lineage XX, and in (Pre-)XDR compared to XXXX (OR XXX). Whilst, we observed increases in risk of transmission in Khyber Pakhtunkhwa province, driven by Peshawar samples (OR XXXX), these may not be entirely robust due to the sampling strategy and high levels of missing location data (**S5 Table**).

A genome-wide association study (GWAS) approach was applied to detect loci linked to transmissibility (**S4 Figure**). It revealed *Rv2102, Rv0914c, nusG, Rv2184c* and *Rv1896c* genes to be the top-five most closely associated with the “transmitted” samples (P<10-8) (**S6 Table, S5 Figure**). Two of these genes are uncharacterised (*Rv2102* and *Rv2184c*) XXXXX betas XXXXX. *Rv0914c* is a putative lipid carrier protein or keto acyl-CoA thiolase, XXXbeta indicates XXX. *Rv1896c* is conserved hypothetical protein linked to S-adenosyl-L-methionine-dependent methyltransferase. Whilst, *nusG* (beta XX; P=) participates in transcription elongation, termination and antitermination. For the *nusG* association, which involves five key mutations (S206G, E186A, R124L, A161V, F232C), we located their position on a phylogenetic tree, and this revealed that only R124L was supported by more than one clade.

The transmission clusters involved six main sub-lineages (1.1.2, 2.2.1, 3, 3.1.2, 4.5, 4.9), and we looked for similar isolates in other populations within the 34k dataset. Ancestral reconstruction revealed

XXXXXXX link to **Figure 2** …… how many similar isolates in other populations XXXX.

### Drug resistance mutations

The common mutations underlying genotypic drug resistance were in known mutations. These included mutations in *rpoB* (D435GFYV 293/460, S450LFWY 308/460) linked to rifampicin, *katG* (S315NIT 374/416) and *fabG1* (-15C>T 52/416) linked to isoniazid, *embB* (G406ASDC 51/385, M306ILV 280/385,

Q497RKP 40/385) linked to ethambutol, *gyrA* linked to fluoroquinolones 9A90V 68/277, S91P 22/277, D94GAHYN 195/277) and *pncA* (118 low frequency <25/258) (**Table 2**).

~~XXXXX add a comment about global context (~~**~~S7 Table~~**~~).~~

The 39 mutations in *katG* occurred globally in 14,040 samples (41.3% of the 34k global dataset), the 39 *rpoB* mutations were found in 7,873 other samples (23.2%) and the 21 *embB* changes occurred in 5,683 other global isolates (16.7%). Other figures for the global context of known mutations are shown in (**S7 Table**).

We investigated isolates that had a DST implying resistance, but no established genetic mutations to explain this phenotype. There were 82 isolates (100/2,430 tests) with such discordance across 8 drugs (amikacin, ciprofloxacin, ethambutol, isoniazid, kanamycin, pyrazinamide, rifampicin, streptomycin), and identified 94 distinct potential genetic markers in candidate genes to explain the discordance (**S2 Table; Table 3; S8 Table**). There were 100/2,430 tests in the case where established genotypic resistance markers were absent in isolates with DST results that implied resistance. The discordance appeared in nine drugs (amikacin (9), capreomycin (2), ciprofloxacin (4), ethambutol (17), isoniazid (25), kanamycin (7), pyrazinamide (24), rifampicin (6), streptomycin (8)). (**S2 Table**). Of the 94 variants, 21 were absence in the 34k global dataset, and had a maximum frequency of 2 (**Table 3; S8 Table**).

For rifampicin resistance, we identified three inframe indels in *rpoB* (1291\_1292insGCC, 1294\_1296del and 1309\_1311del) in three isolates. For isoniazid, several nonsense mutations in *katG* were found, with 3 mutations leading to premature stop codons (W438\*, W204\*, Q36\*) and a frameshift mutation (587\_588insGGT). For ethambutol resistance, variants in the *embA* promoter region (-42CAT>C, -27TA>T-16C>A, -8C>A) and *embB* were observed. For pyrazinamide resistance, several potentially new mutations we found in *pncA*, including three inframe indels (511\_512insTCGCCG, 392\_393insGGT and 451\_462del) a premature stop codon (S18\*), and SNPs in both the coding region (Val180Ala) and the promoter (-7T>G). For streptomycin resistance, several mutations were found in *gid* including a premature stop codon (G71\*), frameshift (102\_102del), and SNPs (A119D, A82P and D67G). These SNPs were found in the 34k global dataset, and likely acquired as the result of homoplasy. The *gid* A119D mutation was present in 15 isolates (ten different sublineages), of which two had DSTs and were resistant. The *gid* A82P mutation was present in three isolates from two different sublineages, but no DST was available for these samples. The *gid* D67G was present in 38 global isolates from five different sublineages. Of these, seven had DST data available with four presenting resistance.

For second line injectables, the *rrs* 878g>a mutation (seen previously 7) was observed in four lineage 3 strains with three independent homoplastic acquisitions, indicating it is unlikely to be strain-specifc. Mutations in *rrs* are generally clustered in two regions with the most common mutations involved with streptomycin resistance being located around position 514 and those involved with resistance to amikacin, kanamycin and capreomycin located around 1401. The *rrs* 878g>a falls between the two mutation hotspots, and of the three strains which had DST data (amikacin and kanamycin) in this study, two were resistant to both amikacin and kanamycin and the other was sensitive to both. For fluoroquinolones, the *gyrA* A288D mutation was found in three lineage 3 isolates and was acquired in each sample independently. One isolate was tested resistant to ciprofloxacin with no known resistance mutation found in the *gyrA* and *gyrB* genes.

## DISCUSSION XXXXXXX NOT LOOKED AT, NEEDS EDITING XXXXX

This study reinforces the value of whole genome sequencing in the context of low resource and high TB burden settings. Our findings on potential transmission among lineages 2 and 4, Pre-XDR and XDR TB, and in Peshawar, as well as drug resistance mutations can inform better epidemiological, clinical, and control decisions in Pakistan and, more generally, provides insight into mutations relevant to drug design. WGS can potentially be particularly useful in countries similar to Pakistan where effective public health surveillance is inadequate due to socio-economic problems - genetic data can fill gaps in data collection, revealing salient connections and variation.

Evidence of increased transmission among lineages 2 and 4 is consistent with previous characterisations of these clades as more transmissible [13][14], and therefore ought to be monitored more closely despite greater prevalence of lineage 3. It is surprising that Pre-XDR and XDR samples were found to be clustered more than expected given the usual fitness cost of drug resistance [15][16]. This however suggests unknown compensatory mutations and ought to be investigated in future work.

Inframe deletions have not been widely reported as a major mechanism of resistance to rifampicin and it is surprising to see the relative high number of these mutations in our dataset.

Nonsense mutations are presumed to lead loss of function in the *katG* gene since it codes for the activator of isoniazid (catalase-peroxidase enzyme), so it is unsurprising to see potentially drug-resistance conferring mutations here. Mutations in the -16 and -8 position of the *embA* promoter have been reported to enhance binding of the *embR* transcription regulator to the promoter and increase expression of *embCAB* operon. The deletions are also presumed to have a similar effect to enhance binding of *embR*, though further experimental evidence would be required in future. The two strains which were resistant to both amikacin and kanamycin also contained a mutation in the promoter region to *eis* (-14C>T). This mutation could potentially explain the resistance to both these drugs and may point to the 878g>a being either increasing the level of resistance to these drugs or potentially conferring resistance to streptomycin (which was not tested for in these samples). The *pncA* gene codes for the activator of pyrazinamide and loss of function leads to resistance. The functional consequence of the premature stop codons and indels are usually severe to protein function and these mutations in our isolates represent highly likely candidates as conferring resistance. Mutations in the promoter region of *pncA* leads to changes in the expression of PncA and resistance [17]. The c.-7T>G mutation is thus also likely to cause resistance. The functional effect of SNPs in the coding region of *pncA* can be harder to predict, however the Val180Ala mutation was reported previously to be associated to pyrazinamide resistance in the CARD database [18].

The incomplete penetrance of the streptomycin-associated D67Gly mutation could be explained by the relative low-level resistance conferred by mutation in *gid* which could be under the critical concentration but still elevated with respect to wild-type.

## METHODS

### Sequence data and processing

WGS were sourced across 6 studies 7–12 (ENA accessions: PRJEB7798, PRJEB10385, PRJEB25972, PRJEB32684, PRJEB43284). Raw reads were trimmed to remove low-quality sequences in Trimmomatic (v0.39) 13, and aligned to the H37Rv reference genome (AL123456) with BWA mem (v0.7.17) 14. SNPs and indels were called using samtools software 15 was processed in gatk GenotypeGVCFs (v4.1.3.0) (gatk.broadinstitute.org). Monomorphic SNPs and variants in non-unique regions of the genome (e.g. *pe/ppe* genes) were excluded. A multi-FASTA format file was created from the filtered SNP file and H37Rv reference fasta using bedtools makewindows (v2.28.0) 16. This multiple alignment was used to construct a phylogenetic tree with IQ-TREE (v1.6.12), involving a general time reversible model with rate heterogeneity set to a discrete Gamma model and an ascertainment bias correction (parameters -m GTR+G+ASC), with 1000 bootstrap samples 17. Pairwise distance matrices were calculated in Plink software (v1.90b4) 18. Drug resistance and lineages were predicted *in silico* from raw sequence data using TB-Profiler (v2.4) 3. The Pakistan analysis results were compared to a global collection of 34k *M. tuberculosis* with WGS and DST data 5.

A cut-off of 10 SNPs difference was established to define transmission clades, and label samples as “transmitted” or “non-transmitted”. A sensitivity analysis was performed to assess the impact of changing the cut-off. Linear mixed models were used perform a GWAS of transmissibility using SNPs, location, drug resistance and adjusting for *M. tuberclusis* (sub-)lineage and outbreak-based population structure, being implemented in GEMMA (v.1.1.2) (<http://www.xzlab.org/software.html>). To identify if samples involved in transmission clades (>10 samples) are similar to others in the global dataset (34k) we constructed phylogenetic trees using FastTree for the relevant sub-lineages (1.1.2, 2.2.1, 3, 3.1.2, 4.5, 4.9). The likelihoods of ancestral locations were conducted with the ape (v5.0) and phytools packages in R.

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**Table 1: Mycobacterium tuberculosis samples (N = 535)**

|  |  |  |  |
| --- | --- | --- | --- |
| Characteristic | Group | N | % |
| Lineage | 1 | 22 | 4.1 |
|  | 2 | 36 | 6.7 |
|  | 3 | 397 | 74.2 |
|  | 4 | 80 | 15.0 |
| Drug resistance status | Sensitive | 60 | 11.2 |
|  | Pre-MDR | 31 | 5.8 |
|  | MDR | 328 | 61.3 |
|  | Pre-XDR | 47 | 8.8 |
|  | XDR | 66 | 12.3 |
|  | Other | 3 | 0.6 |
| Individual drug resistance | Rifampicin | 460 | 86.0 |
|  | Isoniazid | 435 | 81.3 |
|  | Ethambutol | 385 | 72.0 |
|  | Pyrazinamide | 258 | 48.2 |
|  | Streptomycin | 238 | 44.5 |
|  | Ofloxacin | 277 | 51.8 |
|  | Moxifloxacin | 277 | 51.8 |
|  | Levofloxacin | 277 | 51.8 |
|  | Amikacin | 75 | 14.0 |
|  | Kanamycin | 79 | 14.8 |
|  | Capreomycin | 78 | 14.6 |
|  | Ciprofloxacin | 277 | 51.8 |
|  | Ethionamide | 102 | 19.1 |
|  | Para aminosalicylic acid | 10 | 1.9 |
|  | Cycloserine | 2 | 0.4 |
|  | Clofazimine | 1 | 0.2 |
|  | Bedaquiline | 1 | 0.2 |
|  | Fluoroquinolones | 277 | 51.8 |
|  | Aminoglycosides | 75 | 14.0 |
| Collection year | 2003-2005??? | 49 | 9.2 |
|  | 2015 | 364 | 68.0 |
|  | 2016 | 38 | 7.1 |
|  | 2017 | 36 | 6.7 |
|  | 2018 | 21 | 3.9 |
|  | 2019 | 22 | 4.1 |
|  | 2020 | 5 | 0.9 |
| Region | Missing | 372 | 69.5 |
|  | Peshawar | 77 | 14.4 |
|  | Dera Ismail Khan | 25 | 4.7 |
|  | Abbottabad | 13 | 2.4 |
|  | Swat | 13 | 2.4 |
|  | Rawalpindi | 7 | 1.3 |
|  | Hyderabad | 5 | 0.9 |
|  | Karachi | 5 | 0.9 |
|  | Lahore | 5 | 0.9 |
|  | Other | 13 | 2.4 |

**Table 2: Number of samples with known drug resistance-associated mutations**

|  |  |  |  |
| --- | --- | --- | --- |
| Drug | N | Gene | Change [N] |
| Aminoglycosides | 129 | *rrs* | 1401a>g [74], 514a>t [3], 906a>g, [9], 1484g>t [1], 514a>c [47], 905c>g [2], 517c>t [8] |
| Capreomycin | 3 | *tlyA* | 198\_198del [1], N236K [2] |
| Cycloserine | 2 | *alr* | M343T [1], L113R [1] |
| Ethambutol | 385 | *embA* | -12C>T [20], -16C>G [2], -16C>T [13], -11C>A [5] |
|  |  | *embB* | G406A [14], G406S [8], M306I [132], G406D [23], G406C [6], Q497R [21], Q497K [10], Q497P [9], Q853P [2], E405D [1], E504D [2], A313V [1], M306L [21], M306V [127], Y319C [1], Y319S [1], Y334H [2], S347I [1], D354A [7], D1024N [29], D328Y [3] |
| Ethionamide | 54 | *ethA* | 1200\_1201del [1], 1054\_1054del [1], 599\_599del [1], 1261\_1262insCGAGC [1], 1018\_1018del [1], 1047\_1047del [1], 1300\_1301insGT [1], 61\_61del [1], 671\_671del [1], 1290\_1291insC [1], 4326936\_4328449del [5], 4326943\_4328449del [1], 4326944\_4328449del [1], 4327038\_4327099del [1], Q269\* [4], Q347\* [6], L272P [1], L397R [2], T61M [1], 672\_673insG [2], 673\_674insGC [1], 140\_140del [3], 150\_150del [1], 299\_299del [3], 313\_319del [1], 352\_365del [2], 382\_383insG [4], 392\_392del [2], 404\_405insAT [1], 703\_703del [1], 755\_756insGC [2], 825\_825del [1] |
| Fluoroquinolones | 277 | *gyrA* | G88A [1], G88C [3], D89N [4], A90V [68], S91P [22], D94G [128], D94A [20], D94H [4], D94Y [18], D94N [25] |
|  |  | *gyrB* | R446C [1], S447F [5], I486L [1], T500N [3], E501D [4] |
| Isoniazid | 416 | *katG* | 22\_23insA [1], 238\_260del [1], 337\_337del [1], 679\_680insGC [1], 87\_87del [1], 974\_974del [1], 2148451\_2164815del [1], 2149885\_2172950del [1], 2151318\_2157225del [1], 2152294\_2157889del [1], A172V [1], R104Q [1], D259E [1], G297V [1], S140N [2], S315N [8], S315I [1], S315T [365], T275A [2], T380I [3], W191R [2], W328S [1], Y155C [1], Y155S [2], Y337C [1], Y413H [1], V1A [2], 1176\_1177insG [1], 1196\_1197insGA [1], 1284\_1284del [1], 1328\_1328del [1], 1486\_1487insC [1], 2005\_2006insG [2], 58\_58del [1], 58\_59insCT [1], 596\_596del [1], 371\_371del [1], 60\_61insGT [1] |
|  |  | *ahpC* | -54C>T [4], -81C>T [2] |
| Kanamycin | 5 | *eis* | -10G>A [1], -14C>T [3], -37G>T [1] |
| Pyrazinamide | 258 | *pncA* | -11A>C [4], -11A>G [16], -12T>C [1], 108\_108del [1], 13\_14insGA [1], 166\_167insG [1], 194\_203del [1], 206\_207insC [1], 209\_210insACC [1], 226\_236del [1], 230\_231insA [1], 283\_283del [1], 314\_315insG [2], 346\_347insC [2], 377\_378insGA [1], 382\_383insG [1], 391\_392insG [9], 391\_392insGG [18], 393\_394insC [1], 408\_409insT [1], 412\_413insCATT [1], 417\_418insG [3], 424\_425insGA [2], 429\_429del [1], 430\_431insG [1], 438\_439insCG [1], 455\_456insATGGCTTGGC [2], 501\_502insC [1], 53\_53del [1], 61\_62insG [1], 7\_7del [1], 2285437\_2291074del [1], 2288627\_2289103del [2], 2288776\_2288836del [1], 2288825\_2289242del [1], 2289006\_2290299del [1], A134V [1], A143D [1], A146V [2], A171T [4], A3E [2], R140G [4], D12A [3], D136Y [1], D49N [1], D49G [1], D63G [1], D63H [4], C14R [1], C72Y [1], Q10\* [1], Q10R [5], Q10P [5], Q141P [4], G105D [1], G108R [1], G132S [3], G78S [4], G78V [1], G97S [3], H51Q [1], H57P [1], H57Y [4], H71R [3]. H71Y [3], H82R [1], I133T [2], I31S [1], I5T [2], I6M [1], I6T [1], L156P [1], L159R [2], L19R [2], L27P [1], L35P [1], L4S [2], L4W [1], L85P [1], K96R [2], K96E [1], K96T [2], M175T [2], M1T [1], F58L [1], F94L [1], P54L [24], P62L [2], P62S [2], P69R [3], S104R [1], S164P [1],S67P [4], T100I [1], T135P [4], T142A [1],T142M [1], T160P [3], T47P [3], T61P [1], T76I [2], T76P [6], W119R [3], W68\* [1], W68R [2], W68C [3], W68G [2], W68S [1], Y103C [1], Y34S [1], Y41\* [1], V128G [1], V139A [4], V139G [1], V180F [8], V45G [1], V7F [2], V9G [2] |
| Rifampicin | 460 | *rpoB* | 1296\_1297insTTC [2], 1306\_1308del [3], A286V [2], N437Y [1], D435G [5], D435F [2], D435Y [30], D435V [32], Q429H [2], Q432H [1], Q432L [3], Q432K [4], Q432P [1], H445R [9], H445N [8], H445D [6], H445C [2], H445Q [2], H445L [10], H445P [2], H445Y [12], I480V [1], I491F [1], L430R [3], L430P [8], L452P [13], M434I [12], S428R [1], S428T [1], S441Q [1], S441L [2], S450L [293], S450F [2], S450W [10], S450Y [1], S493L [1], T400A [1], T444I [1], V170F [2] |
|  |  | *rpoC* | D747A [1], G332R [6], I491T [14], I885V [1], L527V [5] |
| Streptomycin | 172 | *gid* | 102\_102del [9], 115\_115del [3], 351\_351del [4], 4407713\_4407860del [1], A80P [3], L79S [1] |
|  |  | *rpsL* | K43R [126], K88R [22], K88M [1], K88T [2] |
| INH, Ethionamide | 58 | *fabG1* | -15C>T [52], -17G>T [1], -8T>A [1], -8T>C [4] |
|  |  | *inhA* | I194T [4], I21T[1], S94A [4], I21V [1] |
| PAS | 10 | *folC* | E153A [1], I43S [5], R49W [1], I43T [1] |
|  |  | *thyX* | -16C>T [2] |
| BDQ, CFZ | 1 | *mmpR5* | 192\_193insG [1] |

BDQ bedaquiline; CFZ clofazimine; PAS para aminosalicylic acid

**Table 3: Phenotypically resistance samples (n=82) with variants previously unknown\* to be associated with drug resistance**

|  |  |  |
| --- | --- | --- |
| Drug | Gene | Change [N] |
| Amikacin | *rrs* | -92T>G [1], 878g>a [2] |
| Ciprofloxacin | *gyrA* | A288D [1], A384V [3], E21Q [4], G668D [4], S95T [4] |
|  | *gyrB* | -162C>CG [1], A432V [1], M291I [3] |
| Ethambutol | *embA* | -16C>A [2], -27TA>T [1], -42CAT>C [1], -8C>A [1], **P455Q [1]**, P913S [1], V206M [1], **V534A [1]** |
|  | *embB* | R24P [3], R524H [1], D328H [1], **D328F [2]**, E378A [1], L172R [2], **F330L [1]**, P12Q [1], T546I [1] |
|  | *embC* | R738Q [12], N394D [1], T270I [1] |
|  | *embR* | -207C>G [1], C110Y [1] |
|  | *ubiA* | E149D [1], G268D [1], **F238I [1**], **V188L [1]** |
| Isoniazid | *ahpC* | -52C>A [1], -72C>T [1], -76T>A [4], -76T>C [1], **-76T>G [1]**, -88G>A [23], -93G>A [1] |
|  | *fabG1* | -91G>C [1], -94C>G [1] |
|  | *kasA* | M72I [1] |
|  | *kasA* | **F402I [1]** |
|  | *katG* | **587\_588insGGT [1]**, A122D [1], A348G [1], R463L [23], **R484G [1]**, D189Y [1], **Q36\* [1], G186D [1], G299D [1]**, I103V [1], **L298S [1],** M105I [1], F408S [1], P100T [1], S315T [1], T271I [1], T475I [1], T625K [1], W204\* [1], W438\* [1], **Y197D [1]** |
| Kanamycin | *eis* | L386I [1] |
|  | *rrs* | -92T>G [1] |
| Pyrazinamide | *pncA* | -7T>G [1], 195G>A [1], **392\_393insGGT [1], 451\_462del [1], 511\_512insTCGCCG [1]**, L120R [4], P62T [1], P69T [1], **S18\* [1]**, V130M [1], V180A [1] |
|  | *rpsA* | **-98A>T [1], Q410R [2]** |
| Rifampicin | *rpoB* | -61C>T [5], **1291\_1292insGCC [1]**, 1294\_1296del [1], 1309\_1311del [1] |
| Streptomycin | *gid* | 102\_102del [1], 615T>C [2], A119D [1], A82P [1], D67G [1], G71\* [2] |
|  | *rpsL* | -165T>C [6] |

\* based on TB-Profiler; **Bolded**, if not observed in a large TB Global dataset (35k 5)

**S1 Table: Strain-types**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Lineage | LSP lineage | Spoligotype family | Region of difference no. | N | % |
| 3 | East-African-Indian | CAS | RD750 | 363 | 67.9 |
| 2.2.1 | East-Asian (Beijing) | Beijing-RD181 | RD105;RD207;RD181 | 29 | 5.4 |
| 4.5 | Euro-American | H;T | RD122 | 23 | 4.3 |
| 4.9 | Euro-American (H37Rv-like) | T1 | None | 22 | 4.1 |
| 1.1.2 | Indo-Oceanic | EAI3;EAI5 | RD239 | 14 | 2.6 |
| 3.1.2 | East-African-Indian | CAS;CAS2 | RD750 | 11 | 2.1 |
| 3.1.2.1 | East-African-Indian | CAS2 | RD750 | 10 | 1.9 |
| 3.1.3 | East-African-Indian | CAS | RD750 | 9 | 1.7 |
| 4.8 | Euro-American (mainly T) | T1;T2;T3;T5 | RD219 | 9 | 1.7 |
| 1.2.2.2 | Indo-Oceanic | NA | RD239 | 7 | 1.3 |
| 4.2.2.2 | Euro-American (Ural) | T;LAM7-TUR | None | 7 | 1.3 |
| 2.2.1.1 | East-Asian (Beijing) | Beijing-RD150 | RD105;RD207;RD181;RD150 | 5 | 0.9 |
| 3.1 | East-African-Indian | Non-CAS1-Delhi | RD750 | 4 | 0.7 |
| 2.2.1.2 | East-Asian (Beijing) | Beijing-RD142 | RD105;RD207;RD181;RD142 | 2 | 0.4 |
| 4 | Euro-American | LAM;T;S;X;H | None | 2 | 0.4 |
| 4.1.1.1 | Euro-American (X-type) | X2 | RD183 | 2 | 0.4 |
| 4.1.1.3 | Euro-American (X-type) | X1;X3 | RD193 | 2 | 0.4 |
| 4.2.1 | Euro-American (TUR) | H3;H4 | None | 2 | 0.4 |
| 4.2.2 | Euro-American (Ural) | T;LAM7-TUR | None | 2 | 0.4 |
| 4.6 | Euro-American | T;LAM | None | 2 | 0.4 |
| 4.6.5 | Euro-American | T;LAM | None | 2 | 0.4 |
| 1.1.3.3 | Indo-Oceanic | NA | RD239 | 1 | 0.2 |
| 4.1.1.2 | Euro-American (X-type) | X1 | None | 1 | 0.2 |
| 4.1.2.1 | Euro-American (Haarlem) | T1;H1 | RD182 | 1 | 0.2 |
| 4.6.2 | Euro-American | T;LAM | RD726 | 1 | 0.2 |
| 4.6.2.1 | Euro-American | T3 | RD726 | 1 | 0.2 |
| 4.6.2.2 | Euro-American (Cameroon) | LAM10-CAM | RD726 | 1 | 0.2 |

**S2 Table: Drug-resistant samples according to drug susceptibility tests (DST) and genotypic predictions**

**~~XXXXXX need to complete XXXXX~~**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Drug | DST  N | DST N resistant | DST % resistant | N genotypic resistant\* | % genotypic resistant | DST Susc. Genotypic resist. | DST resist.  Genotypic non-resist. |
| Rifampicin | 487 | 417 | 85.6 | 460 | 86.0 | 6 | 6 |
| Isoniazid | 487 | 411 | 84.4 | 435 | 81.3 | 7 | 25 |
| Ethambutol | 479 | 265 | 55.3 | 385 | 72.0 | 96 | 17 |
| Pyrazinamide | 444 | 189 | 42.6 | 258 | 48.2 | 42 | 24 |
| Streptomycin | 43 | 24 | 55.8 | 238 | 44.5 | 4 | 6 |
| Ofloxacin | 85 | 46 | 54.1 | 277 | 51.8 | 5 | 0 |
| Moxifloxacin | 52 | 4 | 7.7 | 277 | 51.8 | 29 | 0 |
| Levofloxacin | 0 | - | - | 277 | 51.8 | 0 | 0 |
| Amikacin | 110 | 42 | 38.2 | 75 | 14.0 | 0 | 9 |
| Kanamycin | 112 | 44 | 39.3 | 79 | 14.8 | 0 | 7 |
| Capreomycin | 57 | 15 | 26.3 | 78 | 14.6 | 18 | 2 |
| Ciprofloxacin | 37 | 37 | 100.0 | 277 | 51.8 | 0 | 4 |
| Ethionamide | 37 | 6 | 16.2 | 102 | 19.1 | 11 | 0 |
| PAS | 0 | - | - | 10 | 1.9 | 0 | 0 |
| Cycloserine | 0 | - | - | 2 | 0.4 | 0 | 0 |
| Clofazimine | 0 | - | - | 1 | 0.2 | 0 | 0 |
| Bedaquiline | 0 | - | - | 1 | 0.2 | 0 | 0 |
| Linezolid | 0 | - | - | 0 | 0.0 | 0 | 0 |
| Delamanid | 0 | - | - | 0 | 0.0 | 0 | 0 |
| Fluoroquinolones | 174 | 87 | 50 | 277 | 51.8 | - | - |
| Aminoglycosides | 322 | 125 | 38.8 | 75 | 14.0 | - | - |

\* from TB-Profiler; PAS para aminosalicylic acid

**S3 Table: Drug resistance categories by Lineage (L)**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| DR status | L1 N | L1  % | L2  N | L2  % | L3  N | L3  % | L4  N | L4  % | Total  N | Total  % |
| Sensitive | 1 | 4.5 | 0 | 0.0 | 53 | 13.4 | 6 | 7.5 | 60 | 11.2 |
| Pre-MDR | 2 | 9.1 | 0 | 0.0 | 28 | 7.1 | 1 | 1.3 | 31 | 5.8 |
| MDR | 12 | 54.5 | 25 | 69.4 | 242 | 61.0 | 49 | 61.3 | 328 | 61.3 |
| Pre-XDR | 0 | 0.0 | 7 | 19.4 | 25 | 6.3 | 15 | 18.8 | 47 | 8.8 |
| XDR | 5 | 22.7 | 4 | 11.1 | 48 | 12.1 | 9 | 11.3 | 66 | 12.3 |
| Other | 2 | 9.1 | 0 | 0.0 | 1 | 0.3 | 0 | 0.0 | 3 | 0.6 |
| Total | 22 | 100 | 36 | 100 | 397 | 100 | 80 | 100 | 535 | 100.0 |

**S4 Table: Sensitivity analysis of clustering by SNP distance, summary statistics**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| SNP  dist | N  Clust. | N | Median (Max) | L1 | L2 | L3 | L4 | Sens. | Pre MDR | MDR | Pre  XDR | XDR | Other |
| 0 | 28 | 60 | 2 (3) | 2 | 6 | 35 | 17 | 2 | 2 | 17 | 19 | 20 | 0 |
| 1 | 28 | 60 | 2 (3) | 2 | 6 | 35 | 17 | 2 | 2 | 17 | 19 | 20 | 0 |
| 5 | 49 | 136 | 2 (17) | 7 | 16 | 77 | 36 | 2 | 3 | 60 | 29 | 40 | 2 |
| 10 | 55 | 169 | 2 (22) | 7 | 21 | 98 | 43 | 2 | 3 | 87 | 31 | 44 | 2 |
| 15 | 54 | 176 | 2 (22) | 8 | 21 | 103 | 44 | 2 | 4 | 90 | 32 | 46 | 2 |
| 20 | 63 | 200 | 2 (22) | 8 | 24 | 121 | 47 | 2 | 5 | 106 | 35 | 49 | 3 |
| 25 | 68 | 213 | 2 (22) | 8 | 24 | 131 | 50 | 2 | 5 | 118 | 35 | 50 | 3 |
| 30 | 71 | 220 | 2 (22) | 8 | 25 | 137 | 50 | 2 | 5 | 124 | 35 | 51 | 3 |

**S*5* Table: Characteristics of 169 *M. tuberculosis* isolates in 55 clusters with a SNP distance of 10 compared to others ~~XXXXX needs to be completed, collapse variables XXXXXX~~**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | ~~Trans.~~  ~~(169)~~ | ~~%~~ | ~~Non-trans.~~  ~~(366)~~ | ~~%~~ | ~~OR\*~~ | ~~95% CI~~ | ~~P-value~~ |
| ~~Lineage~~ | ~~1~~ | ~~7~~ | ~~4.1~~ | ~~15~~ | ~~4.1~~ | ~~1.00~~ |  |  |
|  | ~~2~~ | ~~21~~ | ~~12.4~~ | ~~15~~ | ~~4.1~~ | ~~3.32~~ | ~~()~~ |  |
|  | ~~3~~ | ~~98~~ | ~~58.0~~ | ~~299~~ | ~~81.7~~ |  | ~~()~~ |  |
|  | ~~4~~ | ~~43~~ | ~~25.4~~ | ~~37~~ | ~~10.1~~ | ~~3.03~~ | ~~()~~ |  |
|  |  |  |  |  |  |  |  |  |
| ~~DR status~~ | ~~Sensitive~~ | ~~2~~ | ~~1.2~~ | ~~58~~ | ~~15.8~~ |  |  |  |
|  | ~~Pre-MDR~~ | ~~3~~ | ~~1.8~~ | ~~28~~ | ~~7.7~~ |  |  |  |
|  | ~~MDR~~ | ~~87~~ | ~~51.5~~ | ~~241~~ | ~~65.8~~ |  |  |  |
|  | ~~Pre-XDR~~ | ~~31~~ | ~~18.3~~ | ~~16~~ | ~~4.4~~ | ~~4.91~~ |  |  |
|  | ~~XDR~~ | ~~44~~ | ~~26.0~~ | ~~22~~ | ~~6.0~~ | ~~5.5~~ |  |  |
|  | ~~Other~~ | ~~2~~ | ~~1.2~~ | ~~1~~ | ~~0.3~~ |  |  |  |
|  |  |  |  |  |  |  |  |  |
| ~~Location~~ | ~~Abbottabad~~ | ~~6~~ | ~~3.6~~ | ~~7~~ | ~~1.9~~ |  |  |  |
|  | ~~DI Khan~~ | ~~13~~ | ~~7.7~~ | ~~12~~ | ~~3.3~~ |  |  |  |
|  | ~~Hyderabad~~ | ~~3~~ | ~~1.8~~ | ~~2~~ | ~~0.5~~ |  |  |  |
|  | ~~Karachi~~ | ~~2~~ | ~~1.2~~ | ~~3~~ | ~~0.8~~ |  |  |  |
|  | ~~Lahore~~ | ~~4~~ | ~~2.4~~ | ~~1~~ | ~~0.3~~ |  |  |  |
|  | ~~Peshawar~~ | ~~46~~ | ~~27.2~~ | ~~31~~ | ~~8.5~~ | ~~4.04~~ |  |  |
|  | ~~Rawalpindi~~ | ~~3~~ | ~~1.8~~ | ~~4~~ | ~~1.1~~ |  |  |  |
|  | ~~Swat~~ | ~~6~~ | ~~3.6~~ | ~~7~~ | ~~1.9~~ |  |  |  |
|  | ~~Other~~ | ~~6~~ | ~~3.6~~ | ~~7~~ | ~~1.9~~ |  |  |  |
|  | ~~Missing~~ | ~~80~~ | ~~47.3~~ | ~~292~~ | ~~79.8~~ |  |  |  |

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Trans. (169) | % | Non-trans. (366) | % | OR\* | 95% CI | P-value |
| Lineage | 1 | 7 | 4.1 | 15 | 4.1 | 1.01 | 0.34, 2.7 | 1e+00 |
| Lineage | 2 | 21 | 12.4 | 15 | 4.1 | 3.31 | 1.58, 7.11 | 6.64e-04 |
| Lineage | 3 | 98 | 58.0 | 299 | 81.7 | 0.31 | 0.2, 0.47 | 2.01e-08 |
| Lineage | 4 | 43 | 25.4 | 37 | 10.1 | 3.03 | 1.81, 5.08 | 1.17e-05 |
| DR status | Sensitive | 2 | 1.2 | 58 | 15.8 | 0.06 | 0.01, 0.25 | 2.88e-08 |
| DR status | Pre-MDR, MDR, Other | 92 | 54.5 | 270 | 73.8 | 0.43 | 0.29, 0.63 | 1.69e-05 |
| DR status | Pre-XDR, XDR | 75 | 44.3 | 38 | 10.4 | 6.86 | 4.28, 11.14 | 5.66e-18 |
| Location | Abbottabad | 6 | 3.6 | 7 | 1.9 | 0.69 | 0.18, 2.54 | 5.72e-01 |
| Location | Dera Ismail Khan | 13 | 7.7 | 12 | 3.3 | 0.88 | 0.34, 2.29 | 8.29e-01 |
| Location | Peshawar | 46 | 27.2 | 31 | 8.5 | 1.48 | 0.76, 2.9 | 2.7e-01 |
| Location | Swat | 6 | 3.6 | 7 | 1.9 | 0.69 | 0.18, 2.54 | 5.72e-01 |
| Location | Other | 18 | 10.8 | 17 | 4.6 | 0.85 | 0.38, 1.93 | 7.05e-01 |

OR odds ratios, \*adjusted for population structure.

**S6 Table**

**Genome-wide association analysis of transmission**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ~~Gene~~ | ~~Function~~ | ~~beta~~ | ~~95% Conf. Int.~~ | ~~P-value~~ |
| ~~Rv2102~~ | ~~Uncharacterised protein~~ | ~~-0.775~~ | ~~-0.54, -1.01~~ | ~~1.9x10~~~~-10~~ |
| ~~Rv0914c~~ | ~~Possible lipid carrier protein or keto acyl-CoA thiolase~~ | ~~-0.781~~ | ~~-0.54, -1.02~~ | ~~2.3x10~~~~-10~~ |
| *~~nusG~~* | ~~Transcription termination protein~~ | ~~0.791~~ | ~~1.04, 0.55~~ | ~~5.8x10~~~~-10~~ |
| ~~Rv2184c~~ | ~~Uncharacterized~~ | ~~-0.699~~ | ~~-0.47, -0.93~~ | ~~2.8x10~~~~-9~~ |
| ~~Rv1896c~~ | ~~Putative S-adenosyl-L-methionine-dependent methyltransferase~~ | ~~-0.643~~ | ~~-0.43, -0.86~~ | ~~8.9x10~~~~-9~~ |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Gene | Function | beta | 95% Conf. Int. | P-value |
| nusG | Transcription termination/antitermination protein NusG | 0.791 | 0.55, 1.04 | 5.82e-10 |
| Rv2307B | Hypothetical glycine rich protein | 0.745 | 0.49, 1 | 1.51e-08 |
| wag31 | Cell wall synthesis protein Wag31 | 0.912 | 0.57, 1.26 | 3.06e-07 |
| proX | Possible osmoprotectant binding lipoprotein ProX | 0.706 | 0.42, 0.99 | 1.28e-06 |
| murA | Peptidoglycan biosynthesis pathway | 0.660 | 0.38, 0.94 | 4.70e-06 |

~~XXXX Beta < 0 implies less or more transmissible????~~

~~Phenotype coding~~

**S7 Table**

**Number of samples with known drug resistance-associated mutations**

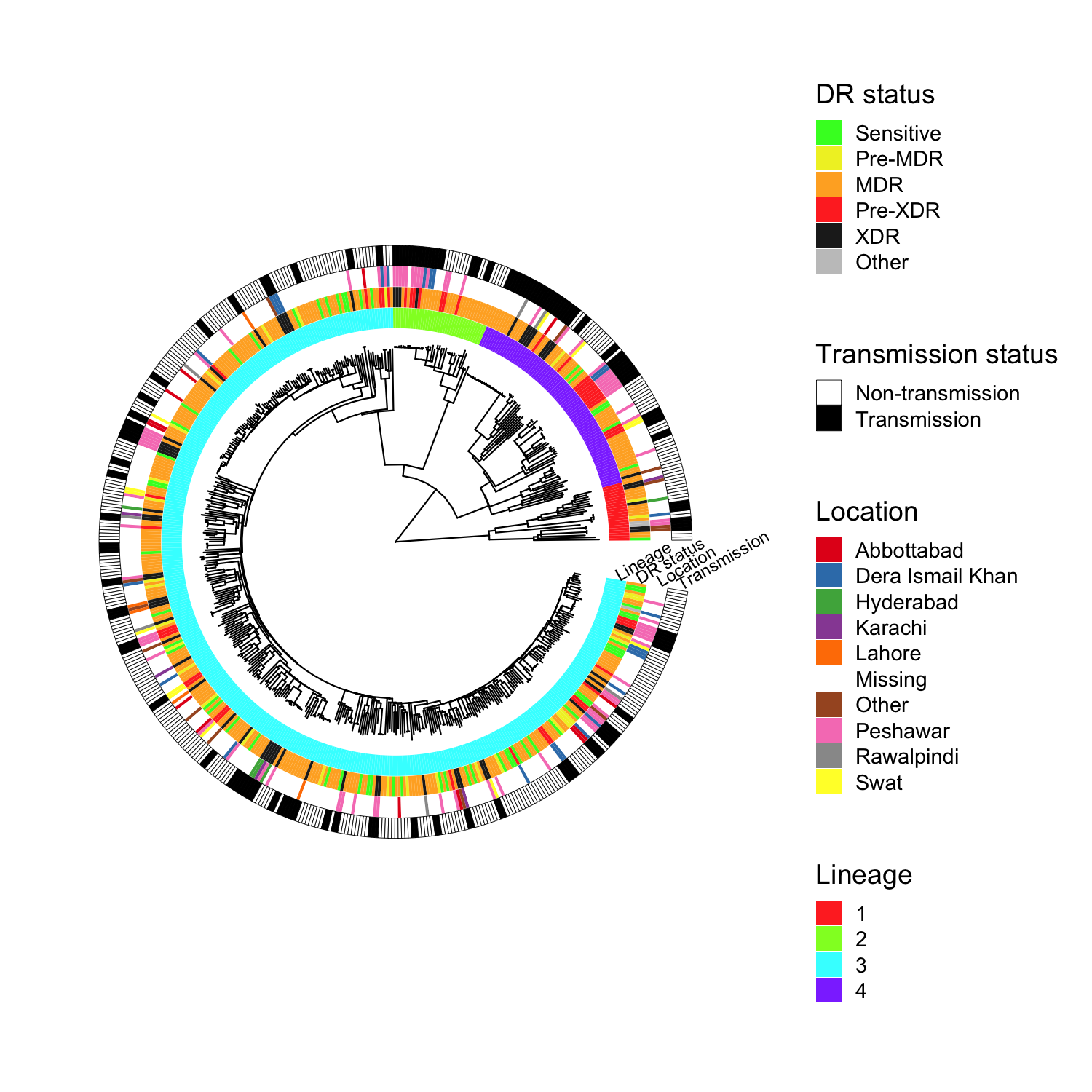
**~~XXXX check if data are % or proportions~~ - % - changed**

**S8 Table**

**Phenotypically resistance samples (n=82) with variants previously unknown to be associated with drug resistance**

**~~XXXX check if data are % or proportions~~ - % - changed**

**Figure 1: A phylogenetic tree for the 535 M. tuberculosis isolates constructed using 37,970 SNPs**

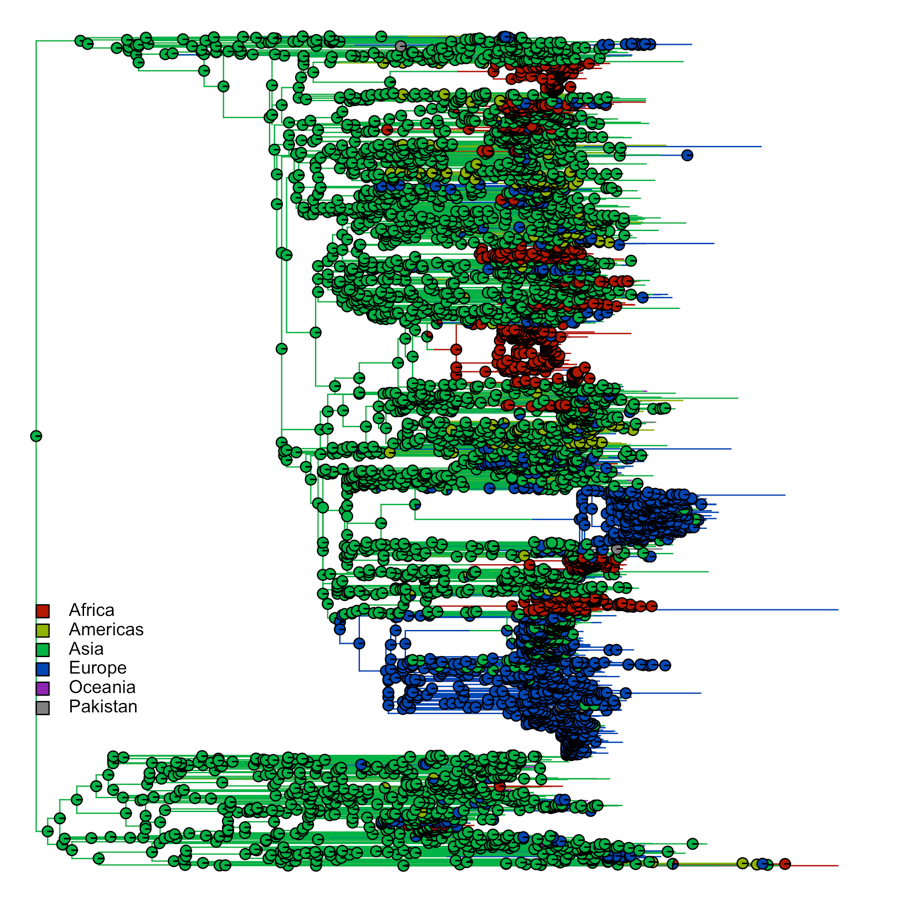


***~~HAVE YOU GOT TRANSMISSION AND NON\_TRANSMISSION LABELLED AROUND THE RIGHT WAY?~~***

**Figure 2: Phylogenetic trees for sub-lineages involving Pakistan samples and closely-related global isolates from previously published datasets. The posterior probability for the ancestral state reconstruction of subregion of sample collection is indicated by the pie charts on the internal nodes.**

1. ***Chart

   Description automatically generated with medium confidence*Sub-lineage 1.1.2 (14 Pakistan, 632 other)**
2. ***Sub-lineage* 2.2.1(29 Pakistan, 6,829 other)**

****

1. ***Sub-lineage 3.1.2* (11 Pakistan, 168 other)**

**Chart

Description automatically generated**

1. ***A picture containing timeline

   Description automatically generatedLineage 3* (363 Pakistan, 2,271 other)**
2. ***Chart

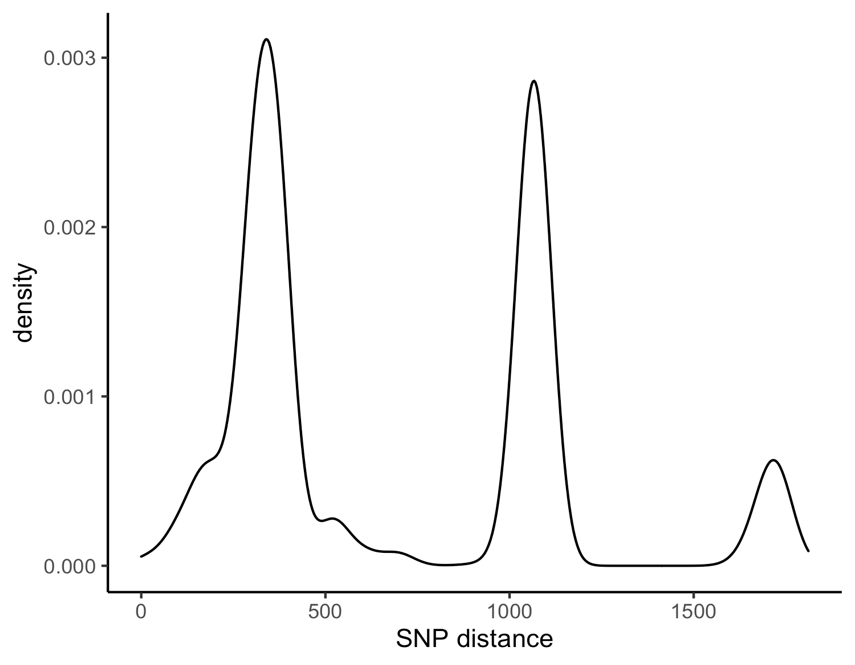
   Description automatically generated with medium confidence*Sub-lineage 4.5 (23 Pakistan, 595 other)**
3. **Chart

   Description automatically generatedSub-lineage 4.9 (22 Pakistan, 253 other)**

Chart

Description automatically generated with medium confidence**S1 Figure: Phylogenetic tree for the 535 M. tuberculosis isolates with individual genomic drug resistance predictions.**

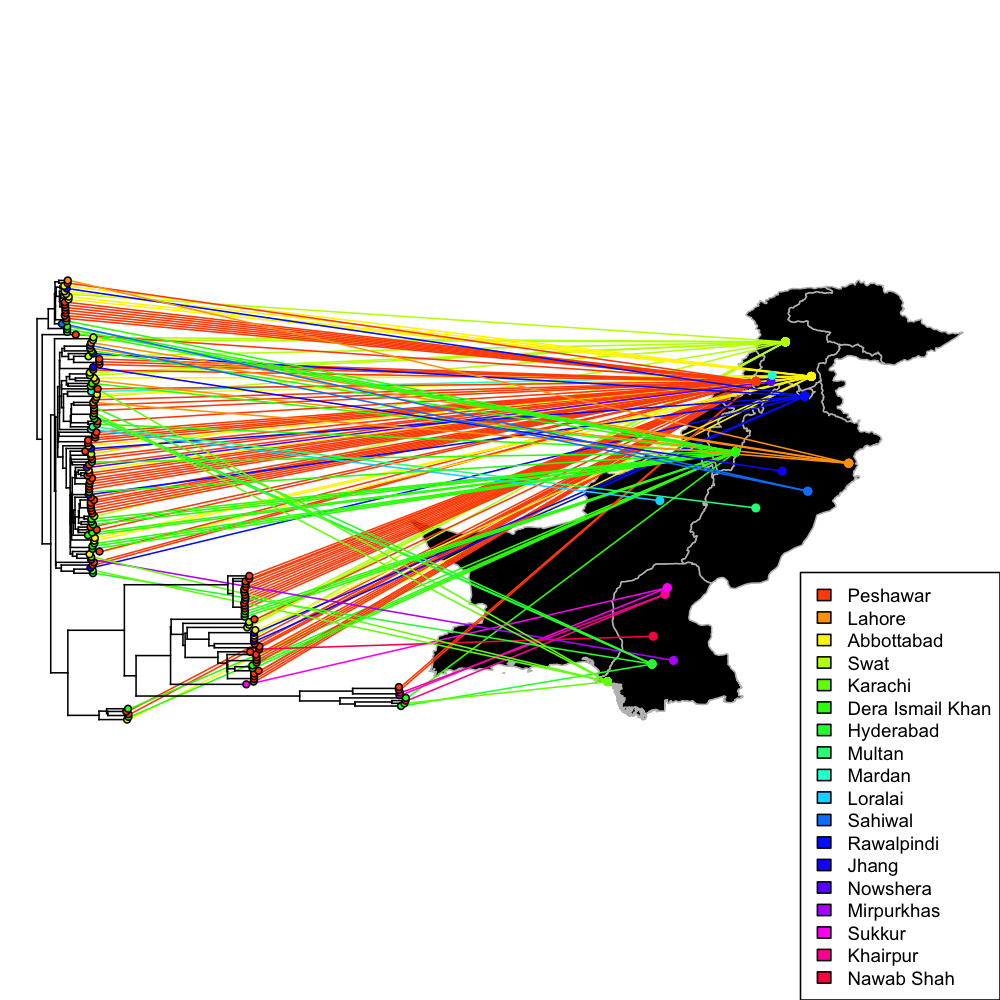
**S2 Figure: SNP distance analyses and clusters (n=535). (top) Density of pairwise SNP differences for all samples; (bottom) number of clustering samples at minimum pairwised SNP difference thresholds**



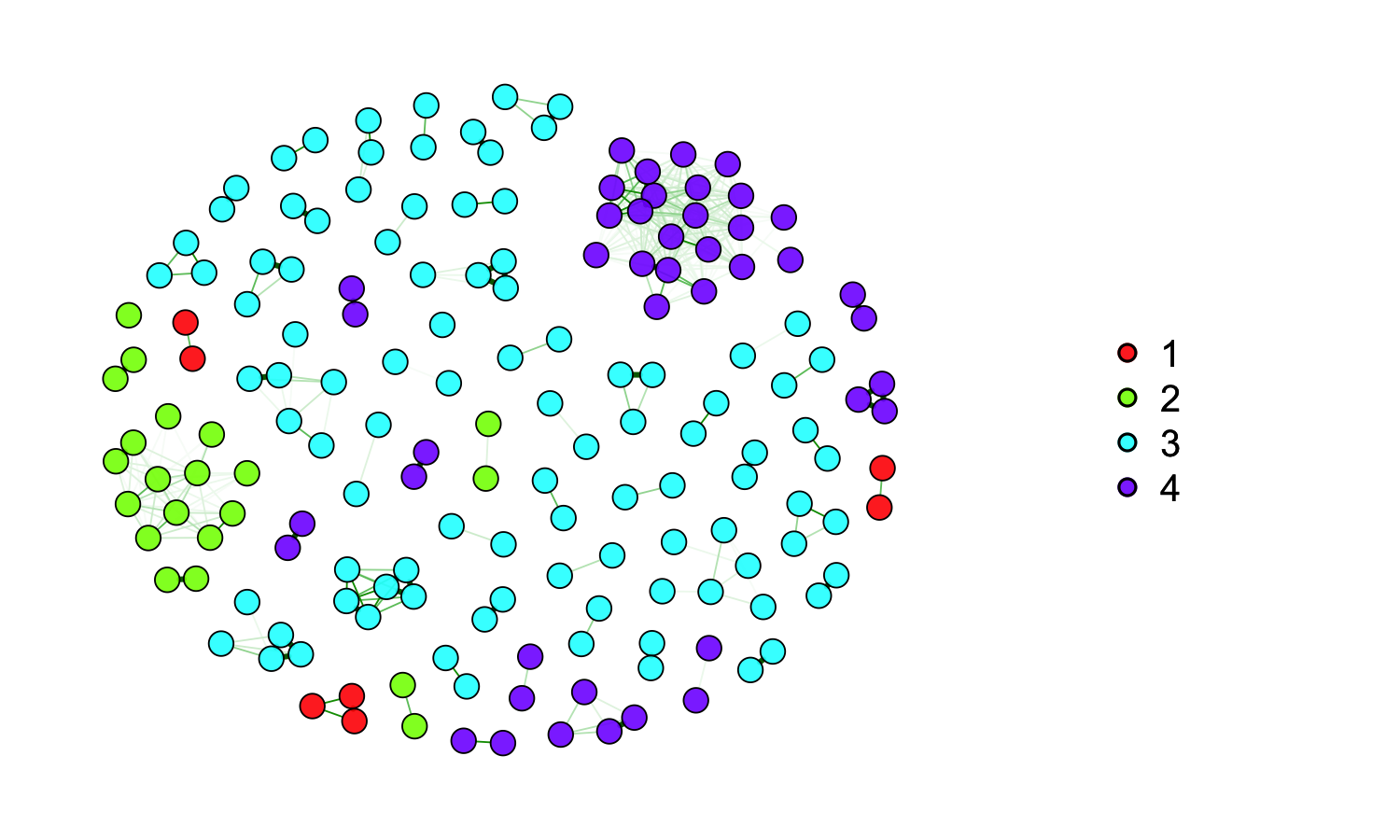
Chart, line chart

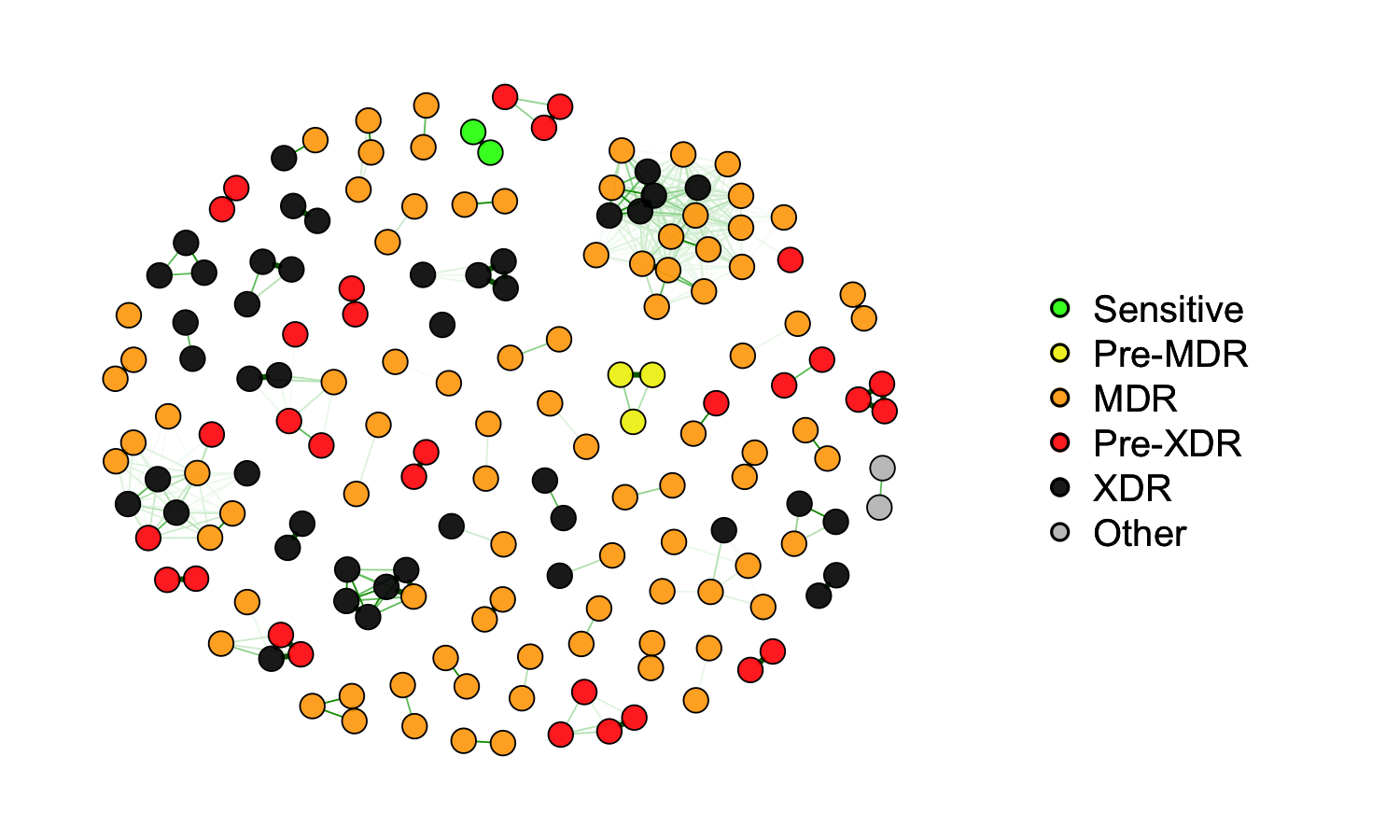
Description automatically generated

**S3 Figure: Locations of samples in the transmission chains (n = 163)**



**S4 Figure: The clusters with isolates at <= 10 SNP distance (n = 169), by lineage (top), drug resistance status (middle), and location (bottom).**







**S5 Figure**

**~~Genome-wide association analysis of transmission. The top-ten scoring genes are labelled.~~**

**Genome-wide association analysis of transmission. The highest five p-value genes with positive betas are labelled.**



~~CHANGE X-AXIS TO LOCATION~~

**S6 Figure**

**Phylogenetic location of mutations in genes compared with location of transmission samples.**

**Diagram

Description automatically generated**

~~XXX~~ ***~~HAVE YOU GOT TRANSMISSION AND NON\_TRANSMISSION LABELLED AROUND THE RIGHT WAY?~~***