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Title: Whole genome sequencing *Mycobacterium tuberculosis* directly from sputum identifies more genetic diversity than sequencing from culture

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Abstract

Background

Repeated culture reduces within-sample *Mycobacterium tuberculosis* genetic diversity due to selection of clones suited to growth in culture and/or random loss of lineages, but it is not known to what extent omitting the culture step altogether alters genetic diversity. We compared *M. tuberculosis* whole genome sequences generated from 39 paired clinical samples. In one sample DNA was extracted directly from sputum then enriched with custom-designed SureSelect (Agilent) oligonucleotide baits and in the other it was extracted from mycobacterial growth indicator tube culture.

Results

DNA directly sequenced from sputum showed significantly more within-sample diversity than that from mycobacterial growth incubator tube culture. This was demonstrated by more variants present as heterozygous alleles (HAs) where both a variant and wild type allele were present within a given sample (p<0.001) and greater within-sample Shannon diversity (p<0.001). Seven genes with high within-sample diversity have previously been identified as targets for positive selection, highlighting their potential role in adaptation to survival within the host and under drug pressure. Resistance associated variants present as HAs occurred in six patients, and in four cases may have provided a genotypic explanation for phenotypic resistance.

Conclusions

Culture-free *M. tuberculosis* whole genome sequencing detects more within-sample diversity and may allow detection of mycobacteria that are not actively replicating.

Key words: Mycobacterium tuberculosis; drug-resistant tuberculosis; whole genome sequencing; sputum; within-patient diversity; heteroresistance

Background

International efforts to reduce tuberculosis (TB) infections and mortality over the last two decades have only been partially successful. In 2017, 10 million people developed TB and it has overtaken HIV as the infectious disease responsible for the most deaths worldwide(1, 2). Drug resistance is a major concern with a steady rise in the number of reported cases globally and rapid increases in some areas(1). Patients with *Mycobacterium tuberculosis* resistant to the first line drugs rifampicin and isoniazid are classed as having multidrug-resistant (MDR) TB and usually treated with a standardised second-line drug regimen for at least nine months, which is also used for rifampicin monoresistance(3, 4). With the emergence of resistance to fluoroquinolones and aminoglycosides (extensively drug-resistant [XDR] TB) there is an increasing need for individualised therapy based on drug susceptibility testing (DST). Individualised therapy ensures patients are treated with sufficient active drugs which can prevent selection of additional resistance, improve treatment outcomes and reduce duration of infectiousness(5-8).

Traditionally, phenotypic culture-based DST was used to identify drug resistance but this is being replaced by rapid genetic tests that detect specific drug resistance conferring mutations. Next generation whole genome sequencing (WGS) of *M. tuberculosis* is being increasingly used in research and clinical settings to comprehensively identify all drug resistance associated mutations(9). *M. tuberculosis* has a conserved genome with little genetic diversity between strains(10), but more detailed analysis of individual patient samples with WGS has identified genetically separate bacterial subpopulations in sequential sputum samples(11-15) and across different anatomical sites(16). This within-patient diversity can occur as a result of mixed infection with genetically distinct strains or within-host evolution of a single infecting strain(17).

Bacterial subpopulations can be detected in clinical samples after sequencing reads are mapped to a reference genome where multiple base calls are detected at a single genomic site. These heterozygous alleles (HAs) at sites associated with drug resistance (resistance associated variants, RAVs) may reflect heteroresistance, where a fraction of the total bacterial population is drug susceptible while the remainder is resistant(18). Identification of genetic diversity within clinical samples is important as it may improve detection of RAVs over currently available genetic tests and consensus-level WGS(18). Identifying RAVs could improve individualised therapy, prevent acquired resistance(12), and give insight into bacterial adaptation to the host.

M. tuberculosis WGS is usually performed on cultured isolates to obtain sufficient purified mycobacterial DNA. However, the culture process can change the population structure from that of the original sample due to genetic drift (random loss of lineages) and/or the selection of subpopulations more suited to growth in culture(19-21), and repeated subculture leads to loss of genetic diversity and heteroresistance(22). Additionally, in the normal course of *M. tuberculosis* infection, some bacteria exist as viable non-culturable persister organisms that are hypothesised to cause the high relapse rate seen following treatment of insufficient duration(23). These organisms are likely to be missed by any sequencing method reliant on culture.

WGS directly from sputum without enrichment is challenging(24). It has recently been improved by depleting human DNA during DNA extraction(25). We have previously reported the use of oligonucleotide enrichment technology SureSelect (Agilent, CA, USA) to sequence *M. tuberculosis* DNA directly from sputum(26) and demonstrated its utility in determining a rapid genetic drug resistance profile(27, 28).

It remains unclear to what extent WGS of cultured *M. tuberculosis* samples underestimates the genetic diversity of the population in sputum samples. One previous study of 16 patients did not identify increased genetic diversity in *M. tuberculosis* DNA sequenced directly from sputum compared to DNA from culture(25), whereas another study of mostly drug susceptible patients showed sequencing directly from sputum identified a slight excess of HAs relative to culture(27). Here we reanalyse heterozygous alleles (HAs) present in that study(27) in addition to newly collected samples from patients with MDR-TB, use a more sensitive analysis to measure overall within-sample genetic diversity and further explore the genomic location of the additional diversity identified.

Results

Patient Characteristics and Drug Susceptibility Testing

Whole genome sequences were obtained for 39 patients from both mycobacterial growth indicator tube (MGIT) culture and direct sputum sequencing. The patients were predominantly of black African ethnicity (80%) and 50% were HIV positive (Table 1). First-line phenotypic drug susceptibility testing (DST) results identified 22 patients with MDR-TB and two with rifampicin monoresistance. In addition there were three isoniazid monoresistant patients and ethambutol resistance was detected in 8 patients. Second-line phenotypic DST was performed for patients with rifampicin-resistant or MDR-TB and identified one case of kanamycin resistance (Table 2).

We observed greater median coverage depth in sputum-derived sequences than MGIT sequences (164.3 vs 136.6, p=0.068). A genotypic susceptibility profile was determined by evaluating MGIT WGS for consensus-level RAVs using a modified version of a publicly available list(29). Genotypic RAVs predicted all rifampicin phenotypic resistance and >90% of isoniazid phenotypic resistance. Ethambutol genotypic RAVs were poorly predictive of phenotypic resistance in line with findings from other studies(30) (Table 2). The patient with kanamycin phenotypic resistance was correctly identified by an *rrs* a1401g RAV. No full phenotypic fluoroquinolone phenotypic resistance was identified, but several colonies from patient F1013 did grow in the presence of ofloxacin (although not enough to be classified as resistant). The consensus sequences from this patient harboured a *gyrB* E501D mutation which is believed to confer resistance to moxifloxacin but not other fluoroquinolones, which may explain the borderline phenotypic DST result(31).

Genetic Diversity

To compare consensus sequences from sputum and MGIT, a WGS consensus sequence-level maximum likelihood phylogenetic tree was constructed (Supplementary Material: Figure 1). Four previously sequenced strains from KwaZulu-Natal were included(32). As expected, all paired sequences were closely related, with a mean difference of 1.30 (range 0-9) single nucleotide polymorphisms (SNPs). Samples from patients F1066 and F1067 were closely related with only one consensus-level SNP separating all four consensus sequences. There was no obvious epidemiological link between these patients (although this study was not designed to collect comprehensive epidemiological information) and they lived 20km apart in Durban. However, both patients were admitted contemporaneously to an MDR treatment facility and sampled on the same day. DNA extraction and sequencing occurred on different runs so the close genetic linkage may represent direct transmission within a hospital setting, a community transmission chain or an unlikely cross-contamination during sample collection.

Having established congruence between sputum and MGIT sequences at the consensus level we then compared genetic diversity by DNA source. We first defined a threshold for calling variants present as heterozygous alleles (HAs) in our entire dataset by using a range of minimum read count frequencies as described in the methods (Figure 1). Below a minimum of five supporting reads there was an exponential increase in the number of HAs identified, which may be indicative of the inclusion of sequencing errors. To reduce this risk, we used a threshold of a minimum of five supporting reads.

Genetic diversity may occur because of within-host evolution or mixed infection. To identify mixed infection we used a molecular barcode(33) to scan all HAs for a panel of 413 phylogenetic SNPs that can resolve *M. tuberculosis* into one of seven lineages and 55 sub-lineages. We found three phylogenetic SNPs among the HAs. In all cases the heterozygous phylogenetic SNP originated from the same sublineage as other SNPs present at 100% frequency, and there were no cases of HAs indicating the presence of more than one lineage or sublineage. This suggests that the genetic diversity identified is mostly or exclusively due to within-host evolution, although there remains a small possibility that mixed infections with two strains from the same sub-lineage could have occurred.

As a first step to comparing diversity between sputum and MGIT sequenced samples we looked at the location of genetic diversity within the *M. tuberculosis* genome. Variants were called in the MGIT and sputum sequences for each patient and classified as present in MGIT only, sputum only or shared (present in both). HAs were widely dispersed across the genome at similar sites in both sputum and MGIT samples but some genes had multiple HAs (Table 3). The highest genetic diversity was found in the ribosomal RNA (rRNA) genes (*rrs* and *rrl*) with 358 HAs, of which 98.6% were only found in sputum-derived sequences.

As rRNA contains regions that are highly conserved across bacteria, it was considered a possibility that SureSelect baits targeting rRNA genes were capturing both *M. tuberculosis* and other bacterial species. To evaluate this, metagenomic assignment was performed on all reads. Sampling reads not assigned to *M. tuberculosis* (i.e. presumed contaminants from other bacteria) and performing a BLAST search against *M. tuberculosis* 16S and 23S rRNA genes indicated that a sizeable proportion of these reads from directly sequenced sputum had a BLAST hit of at least 30 bases (median 11% v 0% of equivalent reads from MGIT sequencing, p<0.001, Supplementary Material: Figure 2). The taxonomic assignment of these reads were indeed typical of genera composing the oral flora, with a high representation of *Actinomyces, Fusobacterium, Prevotella,* and *Streptococcus* (Supplementary Material: Figure 3).

This supported the hypothesis that the baits may enrich rRNA from other organisms so rRNA genes were excluded from further analysis. The difference in diversity between sputum and MGIT sequences can be explained by the selective nature of MGIT media which will enrich *M. tuberculosis* sequences. Importantly the frequency of HAs in other highly diverse genes between sequencing strategies was more balanced (Table 3). Pertinently seven of these genes (*Rv1319c(34)*, *lppB(35)*, *Rv2082(35)*, *ppsA(34, 36)*, *ponA1(36)*, *lppA(37)*, and *pks12(35, 36)*) with high numbers of HAs have been previously identified as highly diverse in comparative genomic studies suggesting the detected HAs are not artefactual. The frequencies at which HAs in these genes were present in MGIT and sputum is shown in Supplementary Material Figure 4.

After confirming the absence of mixed infections and removing rRNA gene sequences we compared the frequency of HAs in sputum and MGIT. There were 2048 variants across the dataset that were present as a HA in either MGIT, sputum or both sequences (Table 4). Variants present in both MGIT and sputum derived sequences were more likely to be present as a HA in the sputum-derived sequence (3.2% v 1.9%, p<0.0001). Of the other variants present as HAs, 821 were unique to direct sputum sequencing and 153 were unique to MGIT sequencing (Table 4). Variants found only in sputum were more likely to be heterozygous than those found only in MGIT or in both (p<0.0001). The distribution of HAs by patient is shown in Figure 2A. HAs found only by one modality were more likely to be SNPs than shared HAs, where the majority were insertions or deletions. The ratio of non-synonymous to synonymous HAs was similar for those that were shared or MGIT only, but was lower for sputum only HAs. Frameshift mutations were most prevalent among shared HAs (Table 4).

To confirm our findings of increased diversity in sputum we calculated mean within-sample diversity (*H*), excluding rRNA genes and repeat regions (see methods). The mean diversity was significantly greater in sputum than MGIT-derived sequences (Figure 2B: 0.116±0.078 v 0.054±0.026 , mean $H_{\text{sputum}}/H_{\text{MGIT}}$ = 2.66, p=3.0 x 10^{-5}).

Genetic diversity in drug resistance genes

HAs in drug resistance-associated regions, including promoters and intergenic regions, were individually assessed. Five of the 39 patients had RAVs present as HAs in at least one gene, which are shown in Table 5. F1002 had three compensatory mutations in *rpoC* present at HAs in both sequences. F1007 had high-level phenotypic isoniazid resistance despite wild type *katG* and *inhA* genes, but did have two *ahpC* promoter variants present as HAs. Neither of these variants are reported frequently but both have been previously associated with resistance in limited numbers of samples(38). As described above F1066 and F1067 were highly related with only one consensus SNP difference between all four sequences. Both had phenotypic high level isoniazid resistance with no consensus-level *katG* or *inhA* mutation, but had frameshift *katG* mutations present as HAs which have the potential to cause resistance(39). F1066 and RF021 had *Rv1979c* and *pncA* mutations respectively at low frequency in sputum only which have the potential to confer phenotypic resistance to clofazimine (*Rv1979c*) and pyrazinamide (*pncA*), although no phenotypic testing was performed for these drugs.

Discussion

In this study we whole genome sequenced DNA from sputum and MGIT culture in paired samples from 39 patients and compared within-patient genetic diversity of the bacterial genome identified from each source. All paired sequences were closely related at the consensus level, and WGS predicted phenotypic drug susceptibility with over 90% sensitivity and specificity for rifampicin and isoniazid in line with published data(40).

The understanding of within-patient *M. tuberculosis* genetic diversity is becoming increasingly important as the detection of rare variants has been shown to improve the correlation between phenotypic and genotypic drug resistance profiles(18) and can identify emerging drug resistance(11, 12). Here we have demonstrated that significantly more genetic diversity is identified by WGS performed directly from enriched sputum than MGIT culture. Not including a culture step avoids the introduction of bias towards culture-adapted subpopulations and the impact of random chance and is also likely to incorporate DNA from viable non-culturable mycobacteria. A reduction in genetic diversity has previously been shown with sequential *M. tuberculosis* subculture(19, 22), but was not confirmed by a study performing WGS directly from sputum(25). However, the 16 paired sputum and MGIT samples compared by Votintseva(25) had a minimum of 5x coverage compared to a minimum 40x coverage in this study, and were likely to contain less genetic material as they were surplus clinical rather than dedicated research samples.

We found that the rRNA genes have high levels of diversity in sputum samples, but believe this is due to non-mycobacterial DNA hybridising to the capture baits — a conclusion borne out by the taxonomic assignment of reads aligning to these genes in common oral bacteria. We therefore exclude these from further analysis, and recommend others using enrichment from sputum do similarly. We use two methods to evaluate within-sample *M. tuberculosis* genetic diversity. First, we demonstrate increased diversity when sequencing directly from sputum with significantly more unique heterozygous alleles (HAs) than sequencing from MGIT culture. We also observed significantly higher genetic diversity in sputum-derived sequences by comparing the Shannon diversity of variable sites across pairs of samples.

Many of the genes with high levels of within-sample diversity are also reported to be targets for convergent evolution, independently accumulating similar mutations on a global scale. This implies that diversity seen on a macroevolutionary scale has a basis in microevolution, and reinforces the importance of accurately characterising the biological function of these genes and their products to aid the identification of new therapeutic targets. Two-thirds of the patients with MDR-TB had already been treated for drug-sensitive TB, and the diversity identified in sputum samples may therefore represent early adaptation to drug pressure. Importantly, as direct sputum sequencing does not rely on live mycobacteria, DNA from recently killed *M. tuberculosis* is likely to also be sequenced, meaning that recent genomic mutations are likely to be represented as HAs.

In four patients, RAVs present as HAs provided a likely genotypic basis for otherwise unexplained phenotypic resistance. Given the small total number of resistance mutations in this study, the excess of heterozygous known RAVs in directly sequenced sputum is not statistically significant. However the presence of heterozygous RAVs in both MGIT and sputum sequences reinforces the biological importance of these mutations.

A limitation of this study is that it can be difficult to distinguish low frequency variants from sequencing error. Ideally low frequency variants could be confirmed by resequencing the same DNA samples. To reduce the risk of sequencing errors yet still identify genetic diversity we used the lowest minimum read threshold at which the number of HAs remained stable. Also, it is reassuring that of all fixed and heterozygous variants called, more than 93% were identified from both DNA sources.

Conclusions

Directly sequencing *M. tuberculosis* from sputum is able to identify more genetic diversity than sequencing from culture. Understanding within-patient genetic diversity is important to understand bacterial adaptation to drug treatment and the acquisition of drug resistance. It also has potential to identify low frequency RAVs that may further enhance genotypic-phenotypic drug resistance correlation.

Methods

Patient enrolment

Adult patients presenting with a new diagnosis of sputum culture-positive TB were included in the study. Patients were recruited in London, UK (n=15) and Durban, South Africa (n=24). All patients recruited in Durban were Xpert MTB/RIF (Cepheid, CA, USA) positive for rifampicin resistance. Two sputum samples were collected prior to initiating treatment, with one inoculated into mycobacterial growth indicator tube (MGIT) culture (BD, NJ, USA) and the other used for direct DNA extraction.

Ethics, Consent and Permissions

All patients gave written informed consent to participate in the study. Ethical approval for the London study was granted by NHS National Research Ethics Service East Midlands–Nottingham 1 (reference 15/EM/0091). Ethical approval for the Durban study was granted by University of KwaZulu-Natal Biomedical Research Ethics Committee (reference BE022/13).

Microbiology

MGIT samples were incubated in a BACTEC MGIT 960 (BD, NJ, USA) until flagging positive. Phenotypic DST data for London samples were those provided to treating hospitals by Public Health England. Phenotypic DST for Durban samples was performed using the solid agar proportion method (Supplementary Material: Methods).

DNA extraction and sequencing

Positive MGIT tubes were centrifuged at 16,000g for 15 minutes and the supernatant removed. Cells were resuspended in phosphate-buffered saline before undergoing heat killing at 95°C for 1 hour followed by centrifugation at 16,000g for 15 minutes. The supernatant was removed and the sample resuspended in 1mL sterile saline (0.9% w/v). The wash step was repeated. DNA was extracted with mechanical ribolysis before purification with DiaSorin Liaison Ixt (DiaSorin, Italy) or CTAB(41). NEBNext Ultra II DNA (New England Biolabs, MA, USA) was used for DNA library preparation.

Sputum samples for direct sequencing were similarly heat killed processed as for MGIT samples. DNA extraction was performed with mechanical ribolysis followed by purification using DiaSorin Liaison Ixt (DiaSorin, Italy) or DNeasy blood & tissue kit (Qiagen, Germany)(41). Target enrichment was performed using SureSelect with a custom-designed bait set providing coverage of the entire *M. tuberculosis* genome as described previously(27). Batches of 48 multiplexed samples were sequenced on a NextSeq (Illumina, CA, USA).

Bioinformatic analysis

Bioinformatic analysis was performed with CLC Genomics Workbench v11.0 (Qiagen, Germany). DNA sequence reads were aligned to an H37Rv reference genome as detailed in the Supplementary Material Methods section (GenBank accession NC_000962.3). All samples had minimum 98% 1x reference genome coverage and mean coverage depth 40x across the genome. Variants falling within or near hypervariable elements were excluded (Supplementary Material: Table 1). A consensus sequence was extracted and used to determine the genotypic drug susceptibility profile. To construct the maximum likelihood phylogenetic tree, variants were called against the reference genome using VarScan v2.3.9 (Supplementary Material: Methods).

For the initial analysis of genetic diversity, variants were included if supported by ≥2 reads, with ≥1 forward and reverse read. The minimum supporting read threshold was increased in a stepwise fashion from 2 to 20. Further analyses were performed on variant tracks where variants were supported ≥5 supporting reads including ≥1 forward and reverse read.

To compare diversity between paired samples, we first mapped reads to the reference genome using bwa mem v0.7.12(42). After verifying all samples had adequate coverage with qualimap(43) (mean ± standard deviation coverage at 10x: 98.0 ± 1.8%) and realigning indels, variants were called with HaplotypeCaller in GATK v3.3.0(44) (Supplementary Material: Methods). The gvcf files were combined for each pair of samples with CombineGVCFs in GATK then screened to remove sites in variable regions and rRNA genes with vcfintersect in vcflib, resulting in 39 paired gvcf files containing allele depths at variable positions for diversity analysis.

Metagenomic assignment

Sequencing reads were classified using Kraken v0.10.6(45) against a custom Kraken database previously constructed from all available RefSeq genomes for bacteria, archaea, viruses, protozoa, and fungi, as well as all RefSeq plasmids (as of September 19th 2017) and three human genome reference sequences(46). The size of the final database after shrinking was 193 Gb, covering 38,190 distinct NCBI taxonomic IDs.

To assess the proportion of contaminating reads that could generate spurious diversity when mapped to *M. tuberculosis* ribosomal genes, we randomly subsampled 100 reads taxonomically assigned as non-*M. tuberculosis* and performed a BLAST search with blastn v2.2.28(47) against rRNA genes from the H37Rv reference genome. We only analysed hits of at least 30 bases.

Statistics

Statistical analyses were performed with Prism v7.0 (Graphpad, CA, USA). The number of HAs in paired samples were compared using a two-tailed Wilcoxon matched-pairs signed rank test. Numbers of HAs found between groups were compared with chi-squared.

Within-sample diversity (H) was calculated using Shannon diversity from the allele frequencies (p). The Shannon index (H_n) expresses the positional entropy at each position (n), with the mean positional entropy (H) indicating greater within-sample diversity:

$$H = \sum_{n} H_n = \sum_{n} \sum_{i \in \{A,C,G,T\}} p_{n,i} \log{(p_{n,i})}$$

To make a fair comparison for each pair of samples, after removing indels, sites were included if they contained a variant in at least one and had a depth coverage ≥30 in both. We found that the depth coverage cutoff had no qualitative effect on the conclusions. The difference in mean within-sample diversity depending on DNA source was compared with a two-tailed Wilcoxon matched-pairs signed rank test.

Abbreviations

DST	drug susceptibility testing
HA	heterozygous allele
MDR-TB	multidrug resistant-tuberculosis
MGIT	mycobacterial growth indicator tube

RAV	resistance-associated variant
rRNA	ribosomal RNA
SNP	single nucleotide polymorphism
TB	tuberculosis
WGS	whole genome sequencing

Declarations

Ethics approval and consent to participate

All patients gave written informed consent to participate in the study. Ethical approval for the London study was granted by NHS National Research Ethics Service East Midlands–Nottingham 1 (reference 15/EM/0091). Ethical approval for the Durban study was granted by University of KwaZulu-Natal Biomedical Research Ethics Committee (reference BE022/13).

Consent for publication

Not applicable

Availability of data and materials

Original fastq files are available at NCBI Sequence Read Archive with BioProject reference PRJNA486713: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA486713/

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Study conception: JB, ASP

Data collection: CB, KB

Analysis and interpretation: CN, LPS, RD, RW

Drafting of manuscript: CN, LPS

Revision of manuscript: FB, JB, ASP

Final approval of manuscript: CN, LPS, RD, RW, KB, CB, JB, FB, ASP

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Tables

	Count / mean	**Range/percentage**
Age	36.7	22 – 64
Male sex	23/37	59.0%
Ethnicity		
Asian	3/35	8.6%
Black African	28/35	80.0%
Caucasian	4/35	11.4%
HIV positive	19/38	50.0%
CD4 count (median)	296.5*	17 – 707
On antiretroviral therapy at time of diagnosis	8/19**	42.1%

Table 1. Baseline patient characteristics for 39 patients (or as otherwise specified where data were missing). *Data missing for 1 patient.

Drug	**Resistance by phenotypic DST**	**Resistance by genotypic DST**	**Genotypic DST sensitivity**	**Genotypic DST specificity**
First-line drugs				
Rifampicin	24/37 (64.9%)	24/39	24/24 (100%)*	24/24 (100%)
Isoniazid	25/37 (67.6%)	24/39	23/25 (92.0%)	23/24 (95.8%)
Ethambutol	8/37 (21.6%)	17/39	8/8 (100%)	8/17 (47.1%)
Second-line drugs				
Ofloxacin	0/24 (0.0%)	1/24	N/A	0/1 (0%)**
Kanamycin	1/24 (4.2%)	1/24	1/1 (100%)	1/1 (100%)

Table 2. Phenotypic and genotypic drug susceptibility testing (DST) results and sensitivity and specificity of genotypic DST relative to phenotypic DST. Phenotypic DST available for first-line drugs for 37 of the 39 patients, and for second-line drugs for 24 patients who demonstrated rifampicin drug resistance. *In two directly-sequenced sputum samples rifampicin RAVs were missed due to low coverage, although they were identified in the corresponding MGIT sample. **This sample had <1% of colonies grow in the presence of ofloxacin, so is categorised as sensitive but may have low-level or heteroresistance to fluoroquinolones (see main text).

	Heterozygous Allele Count				**Gene length**	**Hypothesised gene function**
Gene	**Shared**	**MGIT only**	**Sputum only**	**Total**	**(base pairs)**	
rrs	2	3	180	185	3138	23S rRNA
rrl	0	0	173	173	1537	16S rRNA
Rv1319c	70	1	24	95	1608	Metabolism and respiration
lppB	7	6	10	23	663	Surface lipoprotein
Rv2561	21	0	0	21	294	Unknown function
Rv3424c	1	1	19	21	363	Unknown function
Rv2082	16	1	2	19	2166	Unknown function
ppsA	7	0	11	18	1059	GGPP synthetase (lipid synthesis)
Rv1435c	3	9	6	18	609	Secreted protein
ponA1	5	3	9	17	2037	Cell wall biosynthesis
Rv2277c	2	0	15	17	906	Metabolism and respiration
vapC31	5	0	12	17	429	Possible toxin
Rv2823c	5	2	9	16	2430	Unknown function
lppA	1	3	11	15	660	Surface lipoprotein
pks12	5	5	4	14	12456	MPM synthesis (lipid metabolism)

Table 3. Genes with the most heterozygous alleles (HAs) identified across the entire dataset.

	Shared variants	MGIT only variants	Sputum only variants	Total
All variants vs H37Rv (fixed or heterozygous)				
Total variants	33 153	1162	1217	35532
Variants vs H37Rv present as heterozygous alleles (HAs) only				
Total variants present as HAs (% of total variants)	MGIT 645 (1.9%) Sputum 1074 (3.2%)	153 (13.2%)	821 (67.5%)	2048 (5.8%)
Median HAs per sample	21	3	15	40
Variant type (% all HAs)				
SNP	500 (46.6%)	127 (83.0%)	708 (86.2%)	1335 (65.2%)
MNP	12 (1.1%)	1 (0.7%)	24 (2.9%)	37 (1.8%)
Insertion	303 (28.2%)	8 (5.2%)	31 (3.8%)	342 (16.7%)
Deletion	259 (24.1%)	16 (10.5%)	57 (6.9%)	332 (16.2%)
Replacement	0 (0.0%)	1 (0.7%)	1 (0.1%)	2 (0.1%)
Coding change (% all HAs)				
Non-synonymous	395 (36.8%)	79 (51.6%)	318 (38.7%)	792 (38.7%)
Synonymous	159 (14.8%)	32 (20.9%)	171 (20.8%)	362 (17.7%)
Intergenic	520 (48.4%)	42 (27.5%)	332 (40.4%)	894 (43.7%)
Non-synon/synon ratio	2.48	2.47	1.86	2.19
Stop codon (% of all non-synonymous HAs)	4 (1.0%)	1 (1.3%)	9 (2.8%)	14 (1.8%)
Frameshift (% of all non-synonymous HAs)	185 (46.8%)	19 (24.1%)	47 (14.8%)	251 (31.7%)

Table 4. Variants identified in MGIT derived, sputum derived, or both sequences from paired samples. Values given represent totals for the 39 paired samples. SNP = single nucleotide polymorphism; MNP = multi-nucleotide polymorphism.

Patient ID	**Phenotypic resistance**	**Mutation**	**Frequency (MGIT/sputum)**	**Description**
F1002	Rifampicin	*rpoB* S450L	100%/100%	High confidence resistance mutation
F1002	Rifampicin	*rpoC* G332R(48)	82.6%/21.7%	Putative compensatory mutations
F1002	Rifampicin	*rpoC* L516P(48)	12.7%/7.7%	
F1002	Rifampicin	*rpoC* P1040S(49)	21.7%/12.3%	
F1007	Isoniazid (high)	*ahpC* c-52t(38)	60.0%/50.7%	Rare, have been associated with resistance
F1007	Isoniazid (high)	*ahpC* g-48a(38)	28.6%/30.3%	
F1061	Rifampicin	*rpoB* H445D	16.1%/0.0%*	High confidence resistance mutation
F1061	Rifampicin	*rpoB* S450W	84.4%/0.0%*	High confidence resistance mutation
F1066	Isoniazid (high)	*katG* N218fs	0.0%/6.9%	Possible resistance mutations, not previously described
F1066	Clofazimine – not tested	*Rv1979c* G376D	0.0%/0.5%	
F1067	Isoniazid (high)	*katG* N218fs	10.7%/7.6%	
RF021	Pyrazinamide – testing failed	*pncA* Q122H	0%/2.5%	

Table 5. Resistance-associated variants present as heterozygous alleles (HAs). *These mutations were also present in sputum but due to low coverage of the area (3 and 4 reads respectively) variant calling criteria were not met.

Figure legends

Figure 1. Variation in total number of heterozygous alleles (HAs) identified across all 39 patients in sequences generated from sputum and MGIT depending on minimum supporting read count threshold. Direct sputum samples indicated by red squares, MGIT samples blue circles.

Figure 2. (A) Number of heterozygous alleles (HAs) found in directly sequenced sputum only (sputum), MGIT (MGIT) only or in both samples (shared) by patient. (B) Mean Shannon diversity at variable positions across pairs of samples (*H*) as calculated for MGIT and sputum-derived sequences. Size of point indicates number of variable positions considered (see Methods).

References

1. Global Tuberculosis Report 2018. Geneva: World Health Organization; 2018 2018.

2. Murray CJ, Ortblad KF, Guinovart C, Lim SS, Wolock TM, Roberts DA, et al. Global, regional, and national incidence and mortality for HIV, tuberculosis, and malaria during 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2014;384(9947):1005-70.

3. Dheda K, Gumbo T, Maartens G, Dooley KE, McNerney R, Murray M, et al. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. Lancet Respir Med. 2017.

4. WHO treatment guidelines for drug-resistant tuberculosis. World Health Organization; 2016.

5. Trauner A, Liu Q, Via LE, Liu X, Ruan X, Liang L, et al. The within-host population dynamics of Mycobacterium tuberculosis vary with treatment efficacy. Genome Biol. 2017;18(1):71.

6. Olaru ID, Lange C, Heyckendorf J. Personalized medicine for patients with MDR-TB. J Antimicrob Chemother. 2016;71(4):852-5.

7. Pasipanodya JG, McIlleron H, Burger A, Wash PA, Smith P, Gumbo T. Serum drug concentrations predictive of pulmonary tuberculosis outcomes. J Infect Dis. 2013;208(9):1464-73.

8. Cegielski JP, Kurbatova E, van der Walt M, Brand J, Ershova J, Tupasi T, et al. Multidrug-Resistant Tuberculosis Treatment Outcomes in Relation to Treatment and Initial Versus Acquired Second-Line Drug Resistance. Clin Infect Dis. 2016;62(4):418-30.

9. Satta G, Lipman M, Smith GP, Arnold C, Kon OM, McHugh TD. Mycobacterium tuberculosis and whole-genome sequencing: how close are we to unleashing its full potential? Clin Microbiol Infect. 2017.

10. Sreevatsan S, Pan X, Stockbauer KE, Connell ND, Kreiswirth BN, Whittam TS, et al. Restricted structural gene polymorphism in the Mycobacterium tuberculosis complex indicates evolutionarily recent global dissemination. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(18):9869-74.

11. Sun G, Luo T, Yang C, Dong X, Li J, Zhu Y, et al. Dynamic population changes in Mycobacterium tuberculosis during acquisition and fixation of drug resistance in patients. J Infect Dis. 2012;206(11):1724-33.

12. Merker M, Kohl TA, Roetzer A, Truebe L, Richter E, Rüsch-Gerdes S, et al. Whole genome sequencing reveals complex evolution patterns of multidrug-resistant Mycobacterium tuberculosis Beijing strains in patients. PLoS One. 2013;8(12):e82551.

13. Operario DJ, Koeppel AF, Turner SD, Bao Y, Pholwat S, Banu S, et al. Prevalence and extent of heteroresistance by next generation sequencing of multidrug-resistant tuberculosis. PLoS One. 2017;12(5):e0176522.

14. Black PA, de Vos M, Louw GE, van der Merwe RG, Dippenaar A, Streicher EM, et al. Whole genome sequencing reveals genomic heterogeneity and antibiotic purification in Mycobacterium tuberculosis isolates. BMC Genomics. 2015;16(1):857.

15. Eldholm V, Norheim G, von der Lippe B, Kinander W, Dahle UR, Caugant DA, et al. Evolution of extensively drug-resistant Mycobacterium tuberculosis from a susceptible ancestor in a single patient. Genome Biol. 2014;15(11):490.

16. Lieberman TD, Wilson D, Misra R, Xiong LL, Moodley P, Cohen T, et al. Genomic diversity in autopsy samples reveals within-host dissemination of HIV-associated Mycobacterium tuberculosis. Nat Med. 2016;22(12):1470-4.

17. Ford C, Yusim K, Ioerger T, Feng S, Chase M, Greene M, et al. Mycobacterium tuberculosis--heterogeneity revealed through whole genome sequencing. Tuberculosis (Edinb). 2012;92(3):194-201.

18. Metcalfe JZ, Streicher E, Theron G, Colman RE, Allender C, Lemmer D, et al. Cryptic Micro-heteroresistance Explains M. tuberculosis Phenotypic Resistance. Am J Respir Crit Care Med. 2017.

19. Depledge DP, Palser AL, Watson SJ, Lai IY, Gray ER, Grant P, et al. Specific capture and whole-genome sequencing of viruses from clinical samples. PLoS One. 2011;6(11):e27805.

20. Hanekom M, Streicher EM, Van de Berg D, Cox H, McDermid C, Bosman M, et al. Population structure of mixed Mycobacterium tuberculosis infection is strain genotype and culture medium dependent. PLoS One. 2013;8(7):e70178.

21. Martin A, Herranz M, Ruiz Serrano MJ, Bouza E, Garcia de Viedma D. The clonal composition of Mycobacterium tuberculosis in clinical specimens could be modified by culture. Tuberculosis (Edinb). 2010;90(3):201-7.

22. Metcalfe JZ, Streicher E, Theron G, Colman RE, Penaloza R, Allender C, et al. Mycobacterium tuberculosis subculture results in loss of potentially clinically relevant heteroresistance. Antimicrob Agents Chemother. 2017.

23. Mukamolova GV, Turapov O, Malkin J, Woltmann G, Barer MR. Resuscitation-promoting factors reveal an occult population of tubercle Bacilli in Sputum. Am J Respir Crit Care Med. 2010;181(2):174-80.

24. Doughty EL, Sergeant MJ, Adetifa I, Antonio M, Pallen MJ. Culture-independent detection and characterisation of Mycobacterium tuberculosis and M. africanum in sputum samples using shotgun metagenomics on a benchtop sequencer. PeerJ. 2014;2:e585.

25. Votintseva AA, Bradley P, Pankhurst L, Del Ojo Elias C, Loose M, Nilgiriwala K, et al. Same-day diagnostic and surveillance data for tuberculosis via whole genome sequencing of direct respiratory samples. J Clin Microbiol. 2017.

26. Brown AC, Bryant JM, Einer-Jensen K, Holdstock J, Houniet DT, Chan JZ, et al. Rapid Whole-Genome Sequencing of Mycobacterium tuberculosis Isolates Directly from Clinical Samples. J Clin Microbiol. 2015;53(7):2230-7.

27. Doyle RM, Burgess C, Williams R, Gorton R, Booth H, Brown J, et al. Direct whole genome sequencing of sputum accurately identifies drug resistant Mycobacterium tuberculosis faster than MGIT culture sequencing. J Clin Microbiol. 2018.

28. Nimmo C, Doyle R, Burgess C, Williams R, Gorton R, McHugh TD, et al. Rapid identification of a Mycobacterium tuberculosis full genetic drug resistance profile through whole genome sequencing directly from sputum. Int J Infect Dis. 2017;62:44-6.

29. Coll F, McNerney R, Preston MD, Guerra-Assunção JA, Warry A, Hill-Cawthorne G, et al. Rapid determination of anti-tuberculosis drug resistance from whole-genome sequences. Genome Med. 2015;7(1):51.

30. Walker TM, Kohl TA, Omar SV, Hedge J, Del Ojo Elias C, Bradley P, et al. Whole-genome sequencing for prediction of Mycobacterium tuberculosis drug susceptibility and resistance: a retrospective cohort study. Lancet Infect Dis. 2015;15(10):1193-202.

31. Malik S, Willby M, Sikes D, Tsodikov OV, Posey JE. New insights into fluoroquinolone resistance in Mycobacterium tuberculosis: functional genetic analysis of gyrA and gyrB mutations. PLoS One. 2012;7(6):e39754.

32. Cohen KA, Abeel T, Manson McGuire A, Desjardins CA, Munsamy V, Shea TP, et al. Evolution of Extensively Drug-Resistant Tuberculosis over Four Decades: Whole Genome Sequencing and Dating Analysis of Mycobacterium tuberculosis Isolates from KwaZulu-Natal. PLoS Med. 2015;12(9):e1001880.

33. Coll F, McNerney R, Guerra-Assuncao JA, Glynn JR, Perdigao J, Viveiros M, et al. A robust SNP barcode for typing Mycobacterium tuberculosis complex strains. Nat Commun. 2014;5:4812.

34. Gonzalo X, Drobniewski F. Is there a place for beta-lactams in the treatment of multidrug-resistant/extensively drug-resistant tuberculosis? Synergy between meropenem and amoxicillin/clavulanate. J Antimicrob Chemother. 2013;68(2):366-9.

35. Grandjean L, Gilman RH, Iwamoto T, Koser CU, Coronel J, Zimic M, et al. Convergent evolution and topologically disruptive polymorphisms among multidrug-resistant tuberculosis in Peru. PLoS One. 2017;12(12):e0189838.

36. Farhat MR, Shapiro BJ, Kieser KJ, Sultana R, Jacobson KR, Victor TC, et al. Genomic analysis identifies targets of convergent positive selection in drug-resistant Mycobacterium tuberculosis. Nat Genet. 2013;45(10):1183-9.

37. Phelan JE, Coll F, Bergval I, Anthony RM, Warren R, Sampson SL, et al. Recombination in pe/ppe genes contributes to genetic variation in Mycobacterium tuberculosis lineages. BMC Genomics. 2016;17:151.

38. Ruesen C, Riza AL, Florescu A, Chaidir L, Editoiu C, Aalders N, et al. Linking minimum inhibitory concentrations to whole genome sequence-predicted drug resistance in Mycobacterium tuberculosis strains from Romania. Sci Rep. 2018;8(1):9676.

39. Heym B, Alzari PM, Honore N, Cole ST. Missense mutations in the catalase-peroxidase gene, katG, are associated with isoniazid resistance in Mycobacterium tuberculosis. Mol Microbiol. 1995;15(2):235-45.

40. Coll F, Phelan J, Hill-Cawthorne GA, Nair MB, Mallard K, Ali S, et al. Genome-wide analysis of multi- and extensively drug-resistant Mycobacterium tuberculosis. Nat Genet. 2018;50(2):307-16.

41. Larsen MH, Biermann K, Tandberg S, Hsu T, Jacobs WR. Genetic Manipulation of Mycobacterium tuberculosis. Curr Protoc Microbiol. 2007;Chapter 10:Unit 10A.2.

42. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754-60.

43. Garcia-Alcalde F, Okonechnikov K, Carbonell J, Cruz LM, Gotz S, Tarazona S, et al. Qualimap: evaluating next-generation sequencing alignment data. Bioinformatics. 2012;28(20):2678-9.

44. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010;20(9):1297-303.

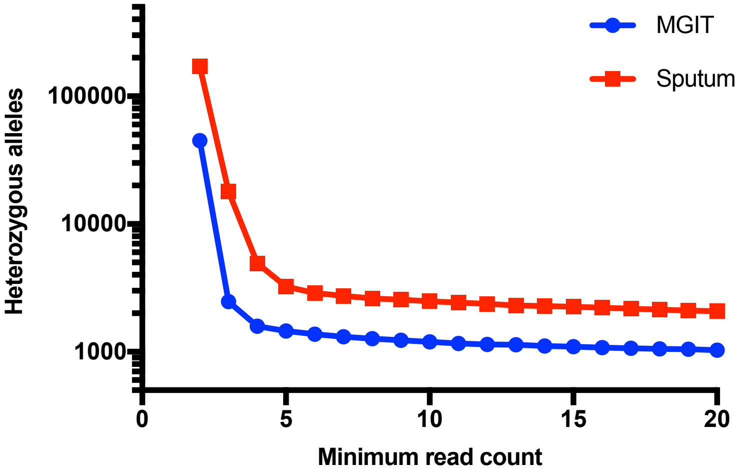
45. Wood DE, Salzberg SL. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 2014;15(3):R46.

46. Lassalle F, Spagnoletti M, Fumagalli M, Shaw L, Dyble M, Walker C, et al. Oral microbiomes from hunter-gatherers and traditional farmers reveal shifts in commensal balance and pathogen load linked to diet. Mol Ecol. 2018;27(1):182-95.

47. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. BMC Bioinformatics. 2009;10:421.

48. Yang C, Luo T, Shen X, Wu J, Gan M, Xu P, et al. Transmission of multidrug-resistant Mycobacterium tuberculosis in Shanghai, China: a retrospective observational study using whole-genome sequencing and epidemiological investigation. Lancet Infect Dis. 2017;17(3):275-84.

49. Eldholm V, Monteserin J, Rieux A, Lopez B, Sobkowiak B, Ritacco V, et al. Four decades of transmission of a multidrug-resistant Mycobacterium tuberculosis outbreak strain. Nat Commun. 2015;6:7119.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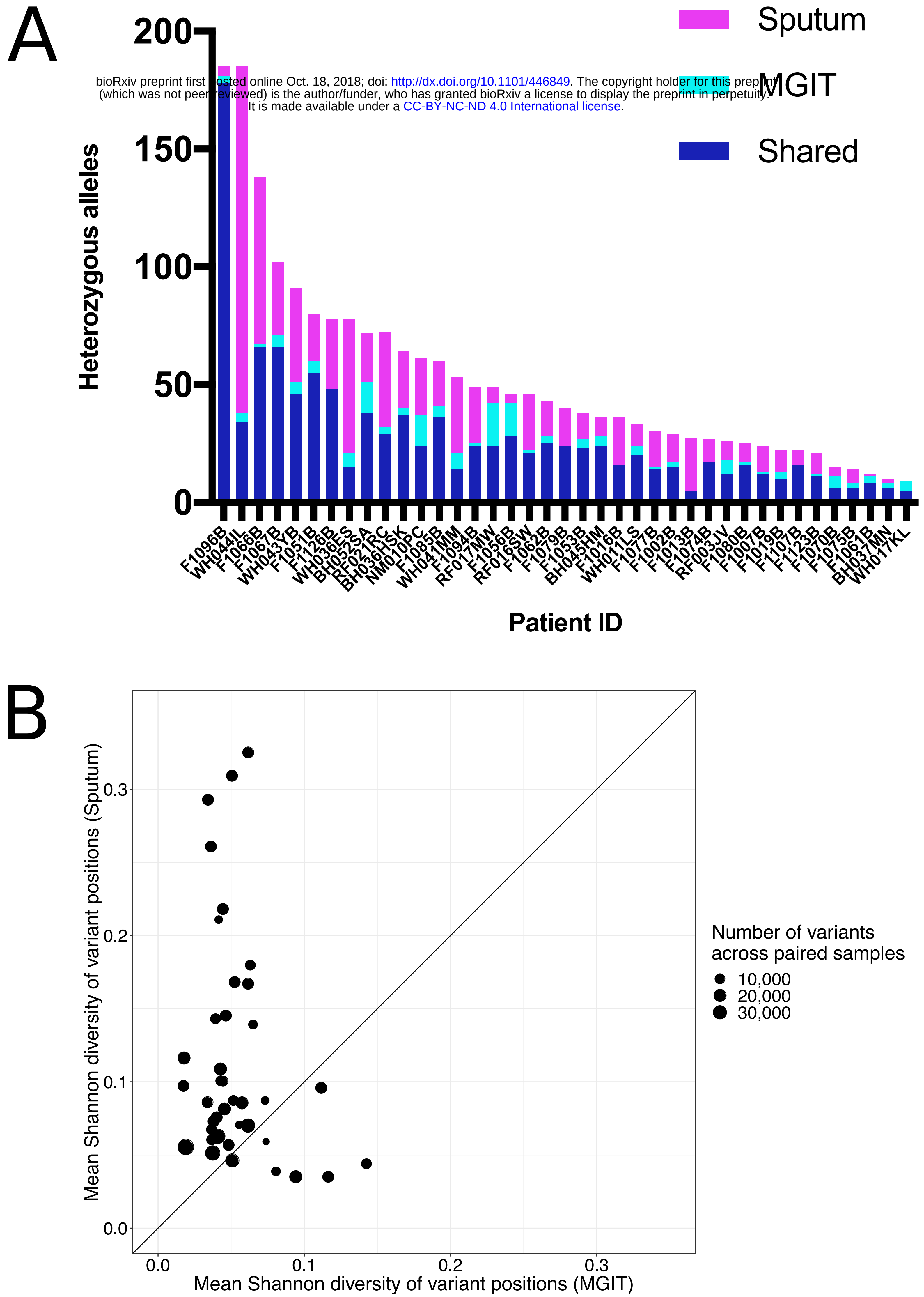
Minimum read count
MGIT
Sputum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Sputum
MGIT
Shared
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