Rapid Whole Genome Sequencing of *M. tuberculosis* directly from clinical samples

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Abstract

The rapid identification of antimicrobial resistance is essential for effective treatment of highly resistant *Mycobacterium tuberculosis* (*M. tb*). Whole genome sequencing provides comprehensive data on resistance mutations and strain typing for monitoring transmission, but unlike conventional molecular tests, this has only previously been achievable from cultured *M. tb*. Here we describe a method utilising biotinylated RNA baits, designed specifically for *M. tb* DNA to capture full *M. tb* genomes directly from infected sputum samples, allowing whole genome sequencing without the requirement of culture. This was carried out on 24 smear-positive sputum samples, collected from the UK and Lithuania where a matched culture sample was available, and two samplesthat had failed to grow in culture. *M. tb* sequencing data was obtained directly from all 24 smear-positive culture-positive sputa, of which 20 were high quality (>20x depth and >90% of genome covered). Results were compared with conventional molecular and culture-based methods, and high levels of concordance were observed between phenotypical resistance and predicted resistance based on genotype. High quality sequence data was obtained from one smear positive culture negative case. This study demonstrates for the first time, the successful and accurate sequencing of *M. tb* genomes directly from uncultured sputa. Identification of known resistance mutations within a week of sample receipt offers the prospect for personalised, rather than empirical, treatment of drug resistant tuberculosis, including the use of antimicrobial-sparing regimens, leading to improved outcomes.

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Introduction

The global incidence of multi, extensively and totally drug resistant tuberculosis has risen over the last decade (1), making it increasingly important to rapidly and accurately detect resistance. The gold standard for antimicrobial resistance testing relies on bacterial culture, which for *M. tb* can take upwards of several weeks. Molecular tests, such as the Xpert (MTB/RIF) and line probe assays, which can be used directly on sputum have improved identification of multi-drug resistant (MDR) *M.tb* but are only able to identify limited numbers of specific resistance mutations (2, 3).

Whole bacterial genome sequencing (WGS) allows simultaneous identification of all known resistance mutations as well as markers with which transmission can be monitored (4). WGS of *M.tb* provides superior resolution over other current methods such as spoligotyping and MIRU-VNTR for strain genotyping (5) and its usefulness in defining outbreaks has been demonstrated (6-9). Currently however, WGS of *M. tb* requires prior bacterial enrichment by culturing and therefore most outbreak studies have been retrospective (6-8). Recently WGS of *M. tb* has been achieved from three-day old MGIT (Mycobacterial growth indicator tube) culture, thus reducing the time from sample receipt to resistance testing to less than a week (10). However,

with the mean time to positive MGIT culture being 14 days (11, 12), most WGS results will not be available for more than two weeks, which is too long a delay before starting therapy. Moreover, the extent to which even limited culture perturbs the original sample composition remains unknown, especially in cases where a patient is suffering from infection with multiple strains, a common occurrence in developing countries where it has been observed in up to 19% of cases (13). As described here we utilised the oligonucleotide enrichment technology SureSelectXT (Agilent) method to obtain the first *M. tb* genome sequences directly from both smear positive and smear negative sputum.

Methods

Samples

A total of 58 routine diagnostic samples from the UK and Lithuania, including 24 smear-positive sputum specimens from pulmonary TB patients and 24 matching cultures (grown on Middlebrook 7H11 plates from the relevant sputum specimens, see below), and 10 sputum samples from patients who had previously been diagnosed with TB and which failed to grow in culture, were analysed. Further details can be found in supplementary table 1. Sputum was visually scored as 1+ to 3+ for acid fast bacilli (AFBs). Sequencing and subsequent analysis were processed blind with respect to smear and resistance results.

Bacteriological methods

Prior to treatment, all sputum specimens were kept frozen at -20 ˚C. Bacteriological culture samples were processed as follows. Samples were decontaminated using N-

acetyl-L-cysteine/NAOH (1% NaOH final concentration) and re-suspended after centrifugation in 2 mL phosphate buffer (pH 6.8). Subsequently, 0.1 ml of the suspension was used for inoculation onto Middlebrook 7H11 media while the remaining suspension was used for the genomic DNA extraction directly from sputum (see below). Plates were incubated at 37 ˚C for at least four weeks or until visible growth was obtained.

DNA extraction from sputum

The bacterial suspension used for inoculation was re-pelleted by centrifugation at 16,000 g. Supernatants were decanted, and pelleted cells re-suspended in 0.3 mL Tris-EDTA (TE), buffer and transferred to sterile 2 mL screw caps tubes containing ~250 µL 0.1 mm glass beads (Becton Dickinson). Microorganisms were heat killed at 80˚C for 50 minutes and then frozen at -20˚C; after thawing the tubes were vortexed for three minutes and centrifuged for five minutes at 16,000 g. The supernatant was transferred to a clean 2mL tube for subsequent DNA purification using the DNeasy Blood and Tissue DNA extraction Kit (Qiagen) as per manufacturer's instructions. Genome copies were measured in the sputum samples using the Artus® *M. tuberculosis* RG PCR Kit (Qiagen), as per manufacturer's instructions.

DNA extraction from cultures

Two loopfuls of *M.tb* growth from Middlebrook 7H11 plates were transferred into 2 mL screw caps tubes containing ~250 µL of 0.1mm glass beads (Becton Dickinson) and 0.3 mL TE buffer. Subsequent processing, genomic DNA extraction and purification were done as described for the sputum samples.

Resistance profiling

All isolates were tested for susceptibility to first line drugs rifampicin (RIF), isoniazid (INH), ethambutol (EMB), pyrazinamide (PZA), and streptomycin (STR). Isolates resistant to at least RIF and INH (i.e. multidrug resistant, MDR-TB) were additionally tested for susceptibility to kanamycin (KAN), amikacin (Amk), ofloxacin (OFL), capreomycin (CAP), ethionamide (ETH), prothionamide (PTH), and par-aminoasalicylate sodium (PAS).

Drug susceptibility testing (DST) was carried out on an automated liquid media-based system Bactec MGIT960 (Becton Dickinson) using standard drug concentrations (in micrograms per millilitre) as follows: STR 1.0; INH 0.1; RIF 1.0; EMB 5.0; PZA 100.0; OFL 2.0; Amk 1.0; CAP 2.5; KAN 5.0; ETH 5.0; PTH 2.5; and PAS 4.0.(14)

Spoligotyping

Spoligotyping was carried out as described previously using membranes with immobilised oligonucleotide probes (Ocimum Biosolutions) (15). For identification of genetic families and lineages, 43-digit binary spoligotyping codes were entered into MIRU-VNTRplus database (www.miru-vntrplus.org) and families identified using similarity search algorithm.

SureSelect^{XT} Target Enrichment: RNA baits design

120-mer RNA baits spanning the length of the positive strand of H37Rv *M. tb* reference genome (AL123456.3),(16) were designed using an in-house Perl script developed by the PATHSEEK consortium. The specificity of the baits was verified by BLASTn searches against the Human Genomic + Transcript database. The custom

designed *M. tuberculosis* bait library was uploaded to SureDesign and synthesised by Agilent Technologies.

SureSelect^{XT} Target Enrichment: Library preparation, hybridisation and Illumina sequencing.

Prior to processing *M.tb* DNA samples were quantified and carrier human genomic DNA (Promega) was added to obtain a total of 3 µg DNA input for library preparation. All DNA samples were sheared for 4x60 seconds using a Covaris S2 (duty cycle 10%, intensity 4 and 200 cycles per burst using frequency sweeping). The samples were then subjected to library preparation using the SureSelect^{XT} Target Enrichment System for Illumina Paired-End Sequencing Library protocol (V1.4.1 Sept 2012). Prior to hybridisation eight cycles of pre-capture PCR was used, and ~750 ng of amplified product was included in each hybridisation (24 hours, 65 ˚C). 16 cycles of post capture PCR was performed, with indexing primers. The resulting library was run on a MiSeq (Illumina) using a 600bp reagent kit, typically in pools of 8 or 10, some sputum smear positive 1+ samples were run in smaller pools to increase coverage. Base calling and sample demultiplexing were generated as standard on the MiSeq machine producing paired FASTQ files for each sample. The raw sequencing data has been deposited on the European Nucleotide archive (upon acceptance of publication). An overview of the process is presented in Supplementary figure 1.

Sequence Analysis

The samples were analysed using a reference based mapping approach implemented in CLC Genomics workbench (v. 7.5). Prior to mapping the reads were

trimmed to remove low quality sequence at the end of reads or adaptor contamination. The reads were mapped against the H37Rv genome (AL123456.3) using default parameters with the addition of a similarity threshold to remove non-*M.tb* reads, by which any reads where at least 90% of the length does not match the reference by at least 90% were discarded. This was required to remove non-*M.tb* reads. Duplicate reads were then removed from the mapped reads, and the average depth of coverage calculated. The percentage of on-target reads (OTR) was calculated by counting the number of reads that were successfully mapped to H37Rv. Any reads that did not map were assumed to be off-target (not *M.tb*).

Bases were called using VarScan (v 2.3.7),(17) applying high stringency parameters including a minimum depth of 4 reads, a minimum average quality of 20, a p value cutoff of 99e-02 and an absence of heterozygosity at a level greater than 10%. A consensus sequence was generated where only called bases were considered, and any bases which failed quality thresholds were called as Ns. To build the phylogeny any variants which were identified in IS elements or the PE, PPE gene families were excluded, as these regions are recognized to be prone to false positive SNP calls.(8) The remaining positions (representing 92% of the genome) were then used to build a maximum likelihood tree using RAxML v 8.0.0(18) with 100 bootstrap replicates.

Genome coverage was calculated by dividing the number of high quality bases successfully called (as per VarScan above) by the reference genome (H37Rv) size. Depth of coverage refers to the number of reads supporting a position.

Calling genotypic resistance

Potential drug susceptibility associated variants were detected using a custom Perl script using positions identified in a curated drug resistance database

(http://pathogenseq.lshtm.ac.uk/rapiddrdata) (19) from bam and bcf files (20). Variants were considered if they were supported by at least 2 forward and reverse reads, had p values of at least 0.05 for strand bias, and 0.001 for read end bias, base quality bias and mapping quality bias as calculated by bcftools (20). A sample was called as genotypically resistant if it had a mutation in over 10% of reads. Any mutation identified in the ribosomal RNA genes were inspected manually to exclude any that may be the result of off-target enrichment of these highly conserved regions. Those that were found on reads that formed distinct haplotypes, where variants were found in close association with other variants on multiple reads were excluded as they likely belonged to non-*M. tb* species.

The analysis was also carried out independently on a customized version of the CLC Genomics Workbench (QIAGEN-AAR), which facilitates a fully-automated pipeline including the steps of trimming, mapping to reference, removal of duplicate mapped reads, variant calling and cross-referencing with the resistance database (described above). Variants called using the automated workflow (using the Low Frequency Variant Detector, CLC Genomic Workbench), were considered significant if the average quality was above 30, a frequency greater than 10% and the forward and reverse read balance was above 0.35. Variants were inspected manually for possible contamination. The runtime when using a standard laptop (Macbook Pro) was on average 1h per sample. The resistance genotypes called were in agreement with those identified using the workflow described above

Results

220 Successful enrichment directly from sputum in both smear positive and negative
221 tuberculosis cases

222 To assess the potential benefits of enrichment strategies for WGS of clinical *M. tb*,
223 we compared the percentage of on-target reads (%OTR), as defined in the methods,
224 and the mean sequencing depth) for two sputum samples, each processed with and
225 without enrichment. The average %OTR for *M. tb* sequenced directly (no-
226 enrichment) from sputum was 0.3%, with 4.6x sequencing depth, compared to with
227 enrichment which generated a %OTR of 82%, with a mean depth of 200x (Figure 1).
228 Although cultured *M. tb* sequenced well with and without enrichment, the former
229 gave greater mean read depth (Supplementary Figure 2). Even coverage across the
230 genome was obtained with no bias observed for particular regions or genes
231 (Supplementary figure 3).

232 Over 98% of the *M. tb* genome was recovered from 20 of 24 (83%) smear-positive
233 culture-positive sputa. Fully complete genomes are not achievable for *M. tb* using
234 short-read sequencing technology, due to the difficulties presented by repetitive
235 regions and the PE and PPE genes (8, 21). Similar levels of genome coverage and
236 sequencing depth were also obtained from the non-enriched matched cultures
237 (Figure 2a). The depth of coverage obtained for four sputum samples was poor
238 (MTB-41: 9x, MTB-42: 14x, MTB-43: 6x and MTB-44: 11x) resulting in a genome
239 coverage of less than 90%. In the case of MTB-43 and MTB-44, which had an input
240 of 1 and <1 genome copies per µl respectively, (these values are out of range of
241 reliable standards as measured by real-time PCR) this is likely to be due to a low
242 pathogen load.

In addition, we were able to enrich and sequence *M.tb* from two smear-positive but culture negative sputum samples (MTB-69 and MTB-73), successfully recovering sequence data from the former with a sequence depth of 8.5x (Figure 2B). We also attempted to sequence eight culture-negative smear-negative sputum samples, which were obtained from previously diagnosed patients (MTB-67-72 and MTB-74 - 76). Surprisingly, for two of these samples, MTB-68 and MTB-76, we obtained high quality *M. tb* sequence data with an average depth of coverage of 9x and 22x, respectively (Figure 2b). For five of the samples we detected low numbers of *M.tb* reads (<1x depth of coverage), which may represent a very low load or residual DNA from dead bacilli. No full length *M. tb* reads were detected in the final sample (MTB-72).

Concordance between genotypes obtained from culture and sputa matched pairs

Using the high quality variable sites called we constructed a maximum likelihood phylogenetic tree. Six samples, with less than 90% genome and SNP position coverage, had an unusual phylogenetic positioning, close to nodes on the tree (Supplementary figure 4), a pattern consistent with a lack of informative sites. With these samples excluded, the resulting robust phylogeny revealed that for all of the matched pairs an identical or near-identical genome was obtained from culture and sputa (Supplementary figure 5).

Concordance between resistance phenotype and genotype

For the 24 matched pairs we sought to identify the genetic resistance determinants which could explain their antibiotic susceptibility profile. Predicted resistance mutations were 100% concordant between sequences obtained from culture and sputa (Table 1), with the exception of the low coverage samples (MTB-41, MTB-42,

MTB-43 and MTB-44) for which we were unable to confidently call variants at many of the targeted loci.

The predicted resistance genotype agreed well with the phenotypic resistance profiles, with a possible resistance conferring mutation being detected in 88% (59/67) of phenotypically resistant cases, and no known resistance mutation in 94% (72/77) of sensitive cases. Two phenotypically pyrazinamide resistant cases (isolated from Patients 7 and 14) both belonging to the URAL lineage were identified as having a large chromosomal deletion (8.64kb) resulting in the removal of the *pncA* gene, the activator of the pro-drug pyrazinamide, plus ten surrounding genes (Supplementary figure 6).

Four samples were phenotypically ethambutol sensitive but had a mutation in codon 306 of the *embB* gene. This mutation has been observed to cause both low and high level resistance to ethambutol, so may lead to a borderline phenotype (22, 23). Patient 15's isolate was phenotypically ethambutol sensitive but had a Q497R mutation in the *embB* gene, which has previously been associated with being sensitive in both clinical isolates (24) and through the construction of isogenic mutants (25) so was discounted. Similarly patient 2's isolate was phenotypically sensitive to isoniazid, but had a G269S mutation in the *kasA* gene, which has also previously been found in sensitive isolates (26). Patient 5's isolate was also phenotypically sensitive to rifampicin notwithstanding a L452P (codon 533 in *E. coli*) mutation in the *rpoB* gene. This mutation has also been associated with both high and low rifampicin resistance in the literature (27). *M. tb* isolated from this patient in the past had been found to be rifampicin resistant suggesting that either this mutation results in a borderline phenotype. Alternatively, a mixture of rifampicin resistant and sensitive strains could have been present in this patient, although this

was not detected in the sequencing data obtained from either the sputum or culture. The remaining eight samples were phenotypically resistant, with an absence of any described or speculative causative genetic mutations. Five of these were phenotypically resistant to second-line drugs for which the genetic basis of resistance is less well understood. These discrepancies highlight that the current limitation on our ability to detect resistance via whole genome sequencing is not the detection itself, but rather lack of data on the genetic correlates of resistance.

Any alleles detected at a low level (<10%), were excluded from this analysis due to the potential problem of carry-over on the sequencing platform which has been previously described (28). Further work will be required to quantify the validity of these mutations, or to assess their clinical significance. In the majority of cases, resistance alleles had reached fixation or near-fixation in both culture and sputa samples, as they were found in 98-100% of the reads. In patient 10 however, significant heterozygosity was detected, with more than one allele being detected at greater than 10% at a single position. A mixture of three different resistance alleles and one sensitive allele were detected within a single codon of the *gyrA* gene (Figure 3). Remarkably almost identical proportions were detected in the corresponding culture sample.

Discussion

Whole genome sequencing of bacteria has been shown to provide comprehensive data on antimicrobial resistance, which could be used to inform antimicrobial

prescribing. However current methods which rely on culturing the organism prior to sequencing are slow and so of limited use in patient management. As a result initial antimicrobial prescribing for resistant *M. tb*, remains largely empirical in the early phase of treatment. Currently MDR *M. tb* can be diagnosed rapidly on the Xpert MTB/RIF system, but a rapid test for extensively drug resistant (XDR) cases is unavailable. As Xpert (MTB/RIF) focuses only on *rpoB* (RIF) mutations unusual resistance patterns where strains are rifampicin sensitive but show other resistance, such as the isoniazid resistant, rifampicin sensitive case included in this study, are missed. Here, we describe the recovery and sequencing of near-complete genomes directly from 81% (21/26) of smear positive sputa, including those staining for low numbers (+1) of Acid Fast Bacilli (AFBs), within a timescale (up to 96 hours) that could allow personalised antimicrobial treatment for both sensitive and resistant cases, including XDR TB.

M. tb is particularly appropriate for the use of diagnostic WGS with enrichment, as, unlike the majority of pathogenic organisms, *M. tb* has a well characterised clonal nature, with relatively low levels of sequence variation and does not undergo recombination or horizontal transfer (29), thus a stable set of oligonucleotide baits can be created and sequence data can be mapped against a reference genome. We have demonstrated that enrichment of *M. tb* provides sequencing data that matches the quality and quantity of data obtained via sequencing from culture. Moreover, we were able to recover high quality *M. tb* sequencing data from one smear-positive and one smear-negative case, both from cases who had received anti-TB therapy and which both failed to grow in culture. However without further clinical information it is difficult to interpret these cases. Smear-positive culture-negative cases are most commonly thought to be due to the on-going persistence of dead bacilli in sputum

samples (30). For this reason, previously treated cases are currently not recommended for use on PCR-based diagnostic systems such as Xpert, that cannot distinguish between dead or live bacilli. Further investigation will be required to assess the suitability of targeted enrichment in the context of different clinical scenarios. There were four smear-positive culture-positive cases where less optimal data were obtained from sputa, although we envisage that sequencing of such low titre samples could be improved through further optimization or increased sequencing depth. It is worth noting these samples were deemed failures based on commonly used SNP calling thresholds employed by others in the field. Further work will be required to robustly establish parameters that are sufficient for clinical use and interpretation, particularly when considering low frequency variants.

Sequencing directly from the clinical sample may reduce any possible biases associated with culture. The overall presence of hetero-resistance in this study was low (one patient), with most resistance conferring mutations observed as close to fixation, i.e. the entire sampled population is resistant. However, in endemic settings mixed infections have been observed to be much more prevalent especially in HIV positive patients (14, 31-33). The detection of these hetero-resistant cases is not only important for our understanding of how resistance evolves, but could impact on clinical management (34). Further studies are required to explore any bias on genetic diversity that may be introduced by culture, particularly in the context of mixed-strain infections.

A disadvantage of the approach presented here is that it is relatively expensive: currently costing approximately $350 (USD) per sample in our laboratory. It also requires skills and machinery currently not available in most microbiological laboratories. An alternative and cheaper rapid sequence based approach would be

to deep sequence total DNA from sputa samples without enrichment.. A recent study found they could recover *M. tb* reads from eight smear and culture positive samples (35). However, in agreement with our study, they obtained a very low depth of coverage (<1x) in the absence of enrichment, so the usefulness of this approach is likely to be limited to detection, and is unlikely to provide the detailed genotype and resistance information that is presented here in a high-throughput manner.

In summary, we have demonstrated whole *M. tb* genome sequencing directly from smear positive, culture positive sputa within a clinically relevant time frame that would enable pro-active patient management. The quality of sequence data allowed us to accurately call mutations that are known to be associated with resistance to first and second line drugs. Furthermore, excluding the need for culture affords new opportunities for biological insights into the evolution of *M. tb* antimicrobial resistance and within-patient evolution.

Figures

Figure 1: Mean coverage and percentage of on target reads (OTR) when sequencing from sputum with and without enrichment for two samples

Figure 2: (A) Depth of coverage obtained for smear positive samples from sputum and culture. (B) Depth of coverage for sputum sequence from smear positive samples which failed to grow. Level of smear positivity is shown, with the remaining being smear negative.

Figure 3: Heteroresistance in gyrA in patient 10. R= resistant allele with suffix indicating codon position, S= absence of resistant allele.

Table 1: Resistance phenotype and genotype of matched pairs. R= a mutation exists at greater than 10%. Low R= mutation in codon 306 of embB gene which is thought to confer low level resistance to ethambutol. Rif = rifampicin, Inh= isoniazid, Emb = ethambutol, Pza = pyrazinamide, Str = streptomycin, Ofl= Ofloxacin (fluoroquinolones), Pas = para-Aminosalicylic acid, Amg = aminoglycosides, Thi = thionamides.

Contributions

The manuscript was written by JMB, ACB and JBreuer with input from all other authors. Bioinformatic analysis and pipeline development was carried out by JMB, JH, DTH, ACB, JZMC, and KEJ. Samples were supplied and processed by FD CR, MM, TDM, VN, AB, RJS, MS HT and JBrown. Enrichment and sequencing was carried out by ACB, and JZMC. ACB, DPD and MTC and members of the PATHSEEK consortium contributed to protocol optimisation. The study was co-ordinated by RW. The study was conceived and initiated by the PATHSEEK consortium and managed by J Breuer, MBM, and GS.

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Competing interests

The authors declare that they have no competing interests. ACB, JH, DTH, JZMC, MBM and GS are or have previously been employed by Oxford Gene Technology and KEJ is employed by QIAGEN-AAR where they received salary and funding.

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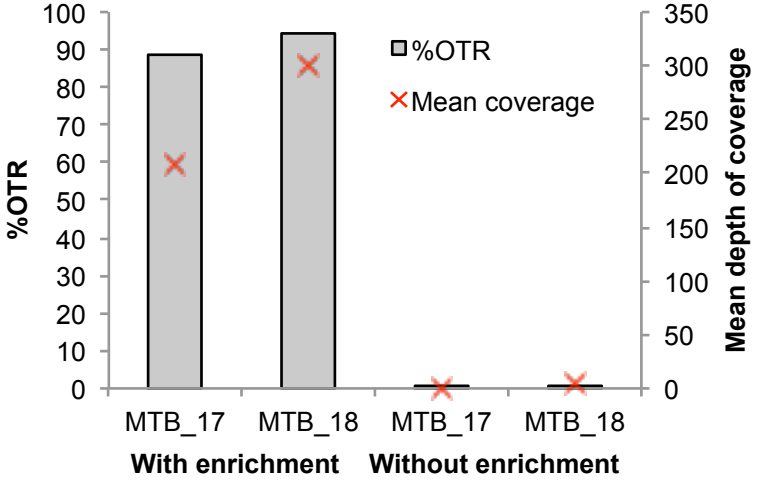
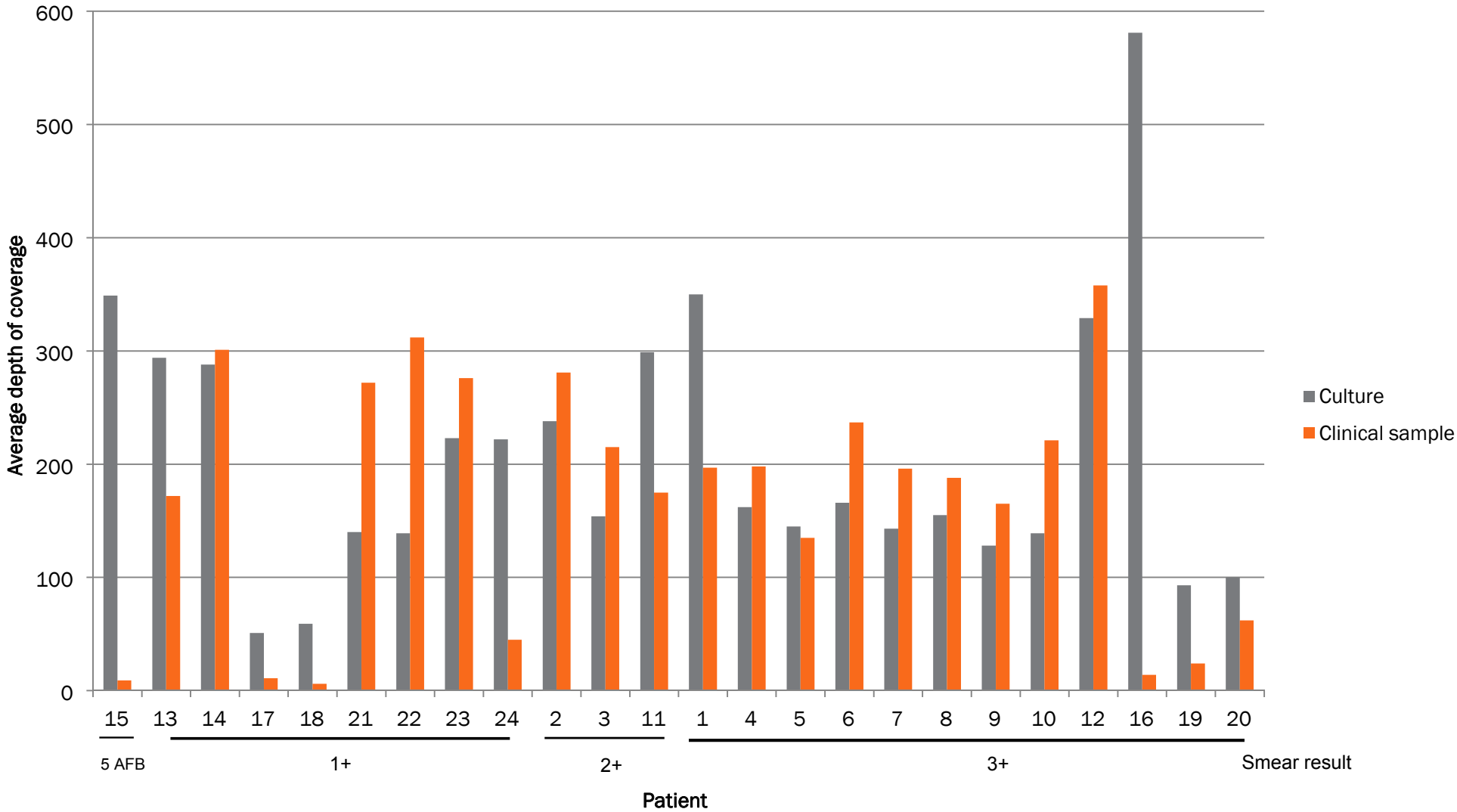

100
90
80
70
60
50
40
30
20
10
0
%OTR
350
300
250
200
150
100
50
0
Mean depth of coverage
%OTR
Mean coverage
MTB_17
MTB_18
MTB_17
MTB_18
With enrichment
Without enrichment

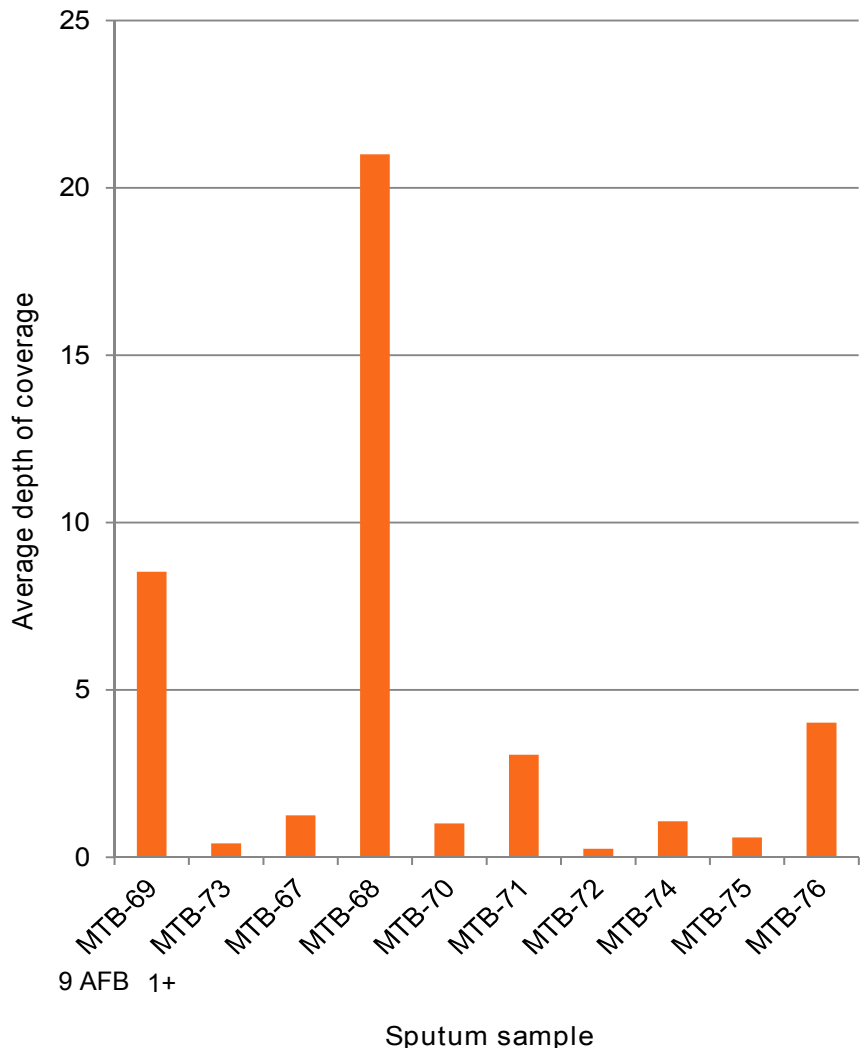
Figure 1

A

Figure 2


600
500
400
300
200
100
0
Average depth of coverage
15 13 14 17 18 21 22 23 24 2 3 11 1 4 5 6 7 8 9 10 12 16 19 20
5 AFB
1+
2+
3+
Smear result
Patient
Culture
Clinical sample

B


25
20
15
10
5
0
Average depth of coverage
MTB-69 MTB-73 MTB-67 MTB-68 MTB-70 MTB-71 MTB-72 MTB-74 MTB-75 MTB-76
9 AFB 1+
Sputum sample

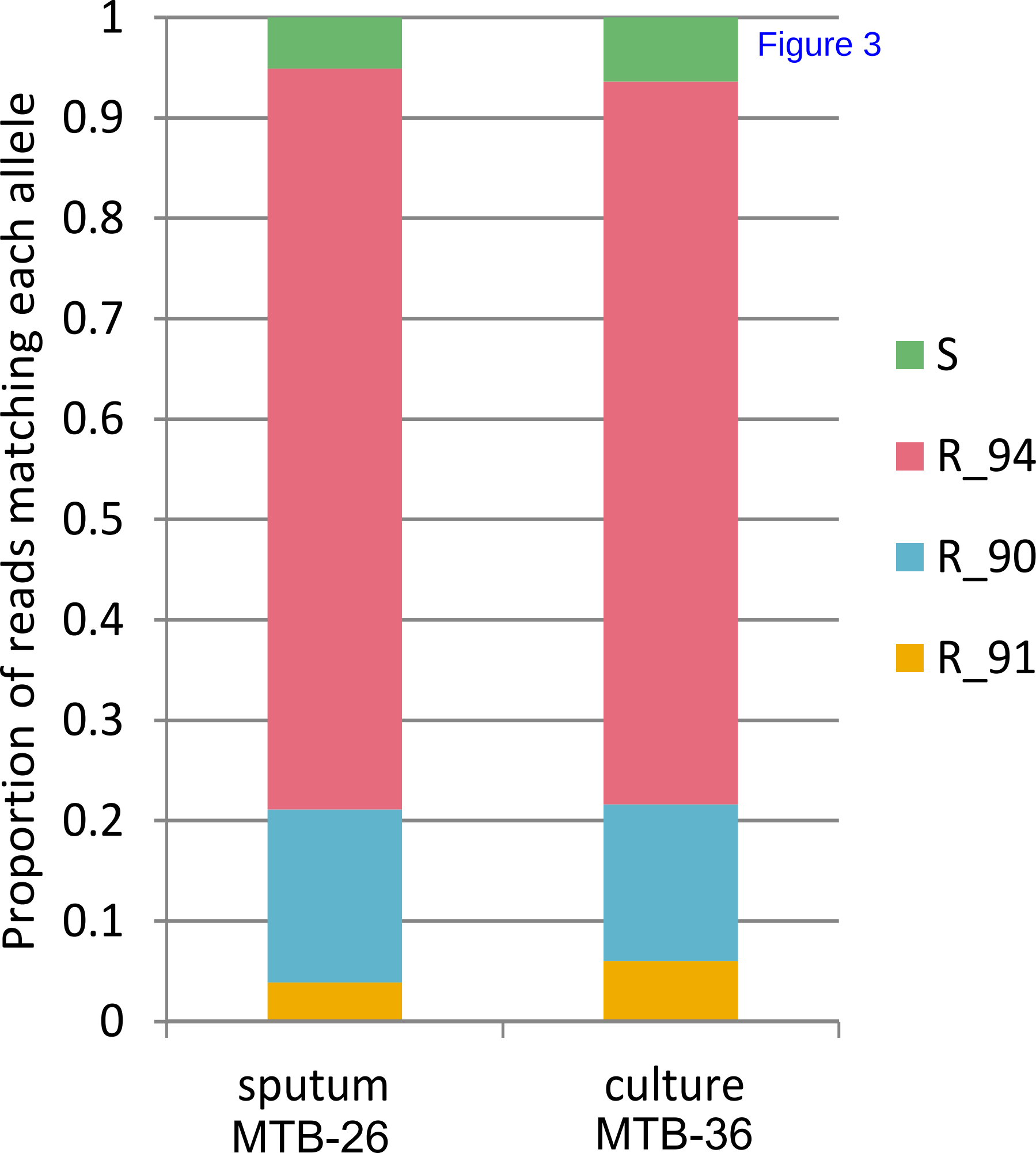


Figure 3
Proportion of reads matching each allele
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
S
R_94
R_90
R_91
sputum
MTB-26
culture
MTB-36

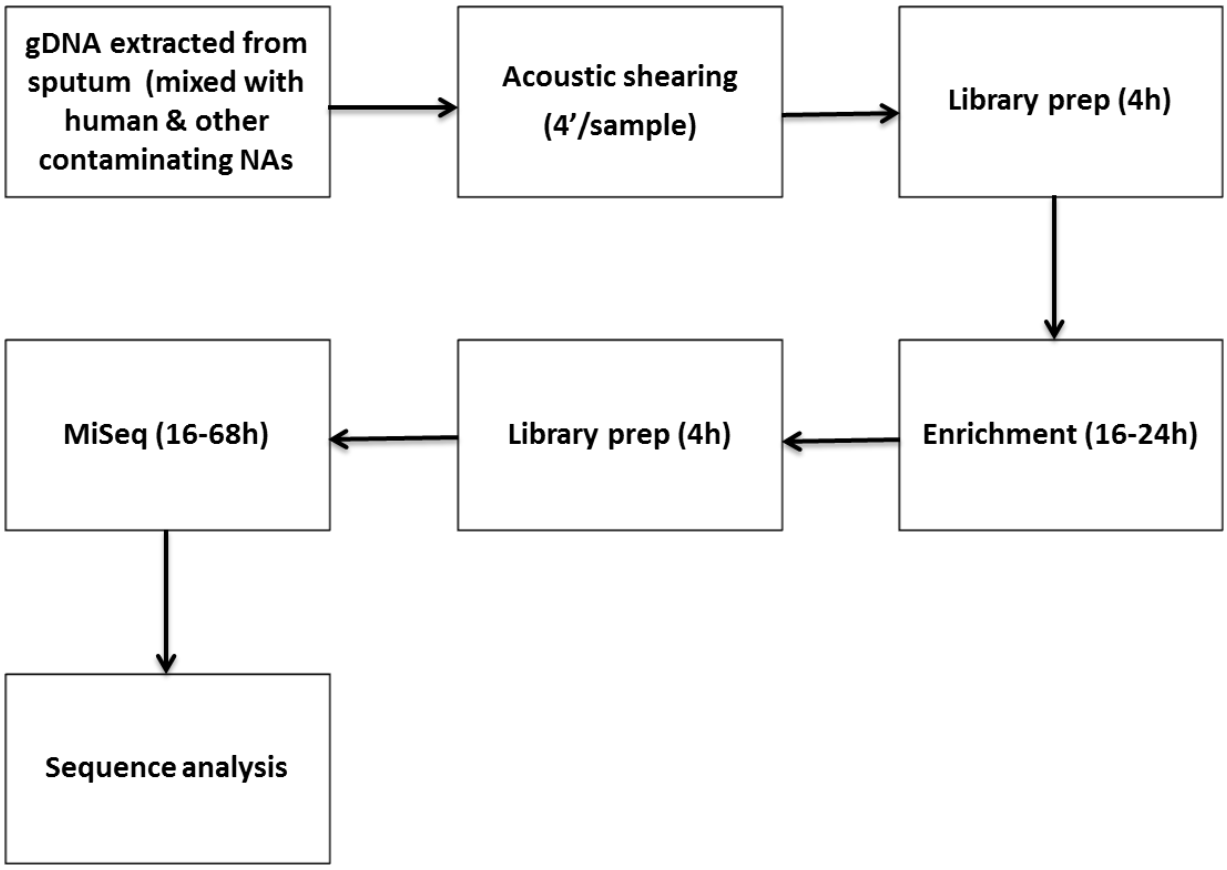
Table 1

Patient	Sputum positivity	Sample	Type	Rif	Inh	Emb	Pza	Str*	Ofl*	Pas*	Amg*	Thi*
1	3+		Culture phenotype	S	S	S	S	NA	NA	NA	NA	NA
		MTB-27	Culture genotype									
		MTB-17	Sputum genotype									
2	2+		Culture phenotype	S	S	S	S	NA	NA	NA	NA	NA
		MTB-28	Culture genotype							R		
		MTB-18	Sputum genotype							R		
3	2+		Culture phenotype	S	S	S	S	NA	NA	NA	NA	NA
		MTB-29WE	Culture genotype									
		MTB-19	Sputum genotype									
4	3+		Culture phenotype	R	R	R	R	R	S	S	R (Kan)	S
		MTB-30WE	Culture genotype	R	R	Low R	R	R			R	
		MTB-20	Sputum genotype	R	R	Low R	R	R			R	
5	3+		Culture phenotype	S	R	S	R	R	R	S	R (Kan & Amk)	R
		MTB-31WE	Culture genotype	R	R	Low R		R	R		R	
		MTB-21	Sputum genotype	R	R	Low R		R	R		R (Kan)	
6	3+		Culture phenotype	R	R	S	R	R	R	S	R (Kan)	R
		MTB-32WE	Culture genotype	R	R	Low R	R	R	R		R	R
		MTB-22	Sputum genotype	R	R	Low R	R	R	R		R	R
7	3+		Culture phenotype	R	R	R	R	R	R	R	R (Cap)	R
		MTB-33WE	Culture genotype	R	R	R		R	R			
		MTB-23	Sputum genotype	R	R	R		R	R			
8	3+		Culture phenotype	R	R	R	R	R	R	R	S	S
		MTB-34WE	Culture genotype	R	R	Low R	R	R	R			
		MTB-24	Sputum genotype	R	R	Low R	R	R	R			
9	3+		Culture phenotype	R	R	R	NA	R	NA	NA	NA	NA
		MTB-35WE	Culture genotype	R	R	R	R	R				
		MTB-25	Sputum genotype	R	R	R	R	R				
10	3+		Culture phenotype	R	R	R	R	R	R	S	S	S
		MTB-36WE	Culture genotype	R	R	Low R	R	R	R			
		MTB-26	Sputum genotype	R	R	Low R	R	R	R			
11	2+		Culture phenotype	S	S	S	S	NA	NA	NA	NA	NA
		MTB-45	Culture genotype									
		MTB-37	Sputum genotype									
12	3+		Culture phenotype	S	R	S	S	NA	NA	NA	NA	NA
		MTB-46	Culture genotype		R							
		MTB-38	Sputum genotype		R							
13	1+		Culture phenotype	R	R	R	R	R	S	S	S	S
		MTB-47	Culture genotype	R	R	R	R	R				
		MTB-39	Sputum genotype	R	R	R	R	R				
14	1+		Culture phenotype	R	R	S	R	R	R	R	S	S
		MTB-48	Culture genotype	R	R	Low R	R	R				
		MTB-40	Sputum genotype	R	R	Low R	R	R				
15	5 AFB		Culture phenotype	R	R	S	R	R	R	NA	S	NA
		MTB-49	Culture genotype	R	R		R	R	R	R		
		MTB-41	Sputum genotype	Below level of detection								
16	3+		Culture phenotype	S	R	S	S	NA	NA	NA	NA	NA
		MTB-50	Culture genotype		R							
		MTB-42	Sputum genotype		R							
17	1+		Culture phenotype	R	R	S	S	NA	S	NA	S	NA
		MTB-51	Culture genotype	R	R	Low R				R		
		MTB-43	Sputum genotype	Below level of detection								
18	1+		Culture phenotype	S	S	S	S	NA	NA	NA	NA	NA
		MTB-52	Culture genotype					R				
		MTB-44	Sputum genotype					R				
19	3+		Culture phenotype	S	S	S	S	NA	NA	NA	NA	NA
		MTB-60	Culture genotype							R		
		MTB-53	Sputum genotype							R		
20	3+		Culture phenotype	S	S	S	S	NA	NA	NA	NA	NA
		MTB-61	Culture genotype									
		MTB-54	Sputum genotype									
21	1+		Culture phenotype	S	S	S	S	NA	NA	NA	NA	NA
		MTB-63	Culture genotype									
		MTB-55	Sputum genotype									
22	1+		Culture phenotype	S	S	S	S	NA	NA	NA	NA	NA
		MTB-64	Culture genotype									
		MTB-56	Sputum genotype									
23	1+		Culture phenotype	S	S	S	S	NA	NA	NA	NA	NA
		MTB-65	Culture genotype									
		MTB-57	Sputum genotype									
24	1+		Culture phenotype	R	R	S	S	NA	S	NA	S	S
		MTB-66	Culture genotype	R	R	Low R				R		
		MTB-59	Sputum genotype	R	R	Low R				R		

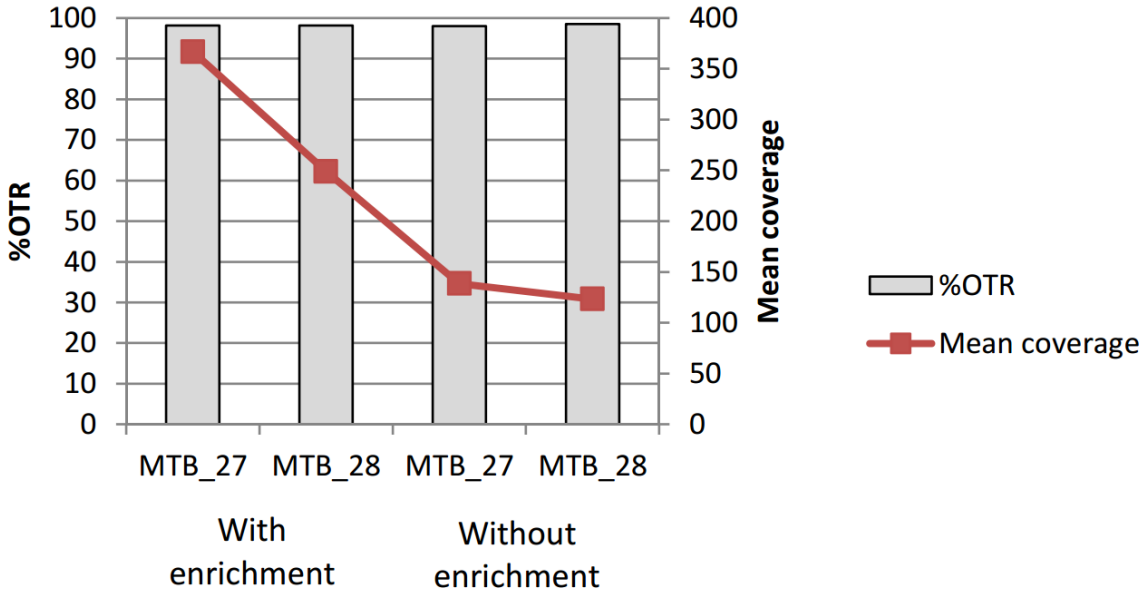
Supplementary table 1: Available details of samples sequenced in this study.

Excel spreadsheet

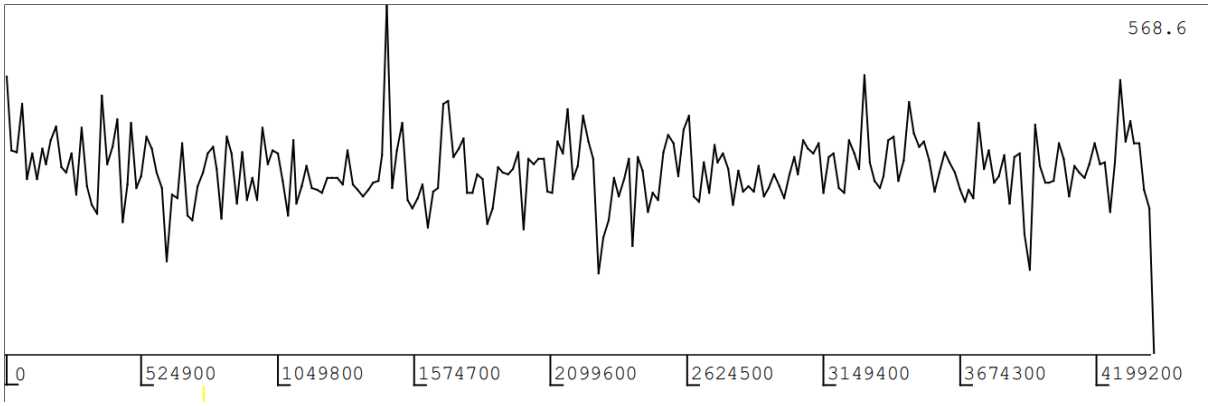
Supplementary Figure 1: Flow diagram for preparation of M. tb samples for enrichment based WGS from receipt of extracted genomic DNA to final data report. Times given are based on 16 samples processed manually or 96 samples processed using automation; Enrichment- we have seen comparable data from 16h vs 24h enrichment; MiSeq times are depending on MiSeq cartridge and chemistry used (2x75bp v3 run = ~16h, 2x300bp v3 run = ~68h)



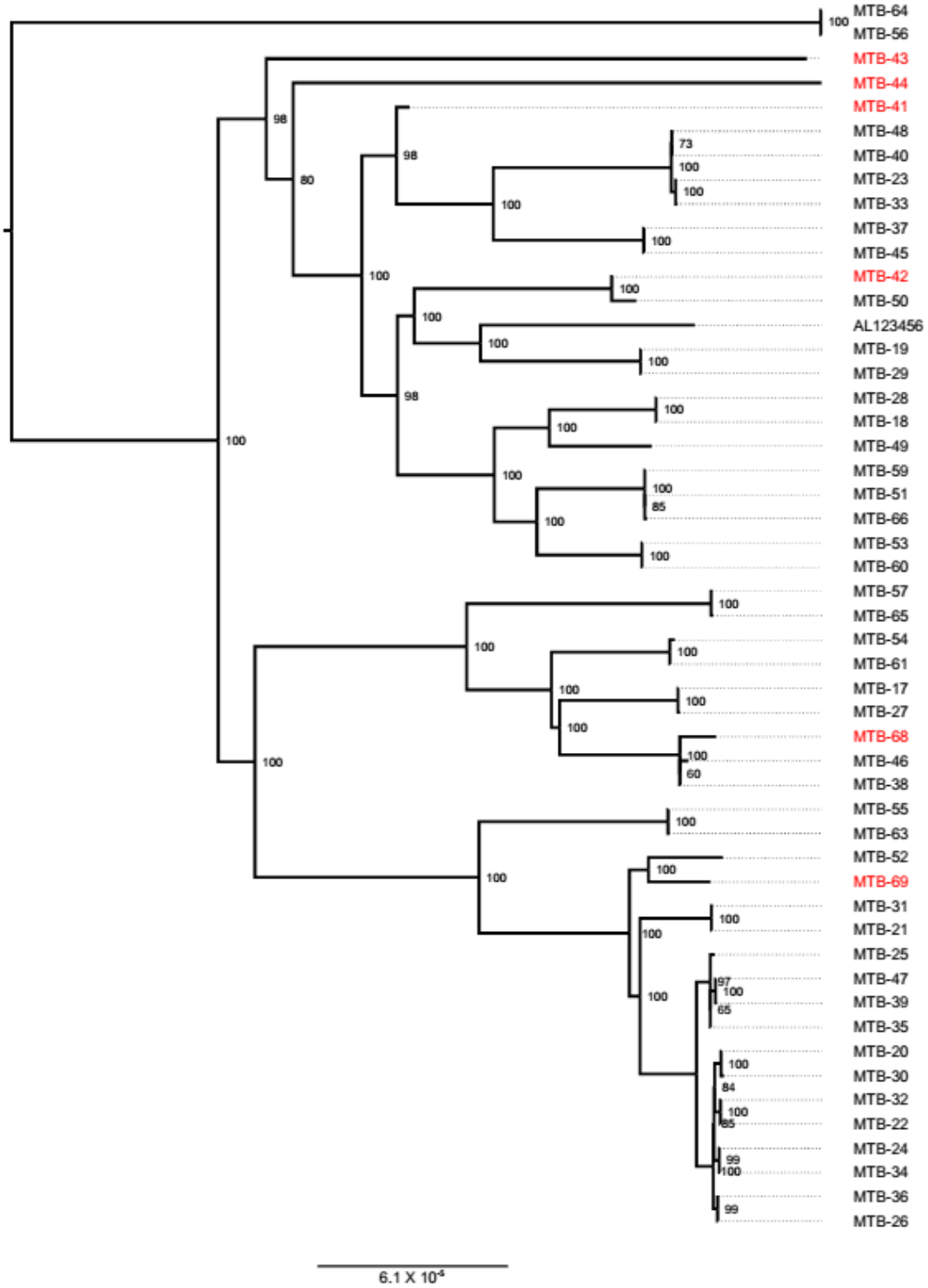
Supplementary Figure 2: Comparison of sequence results from culture with and without enrichment for two samples.



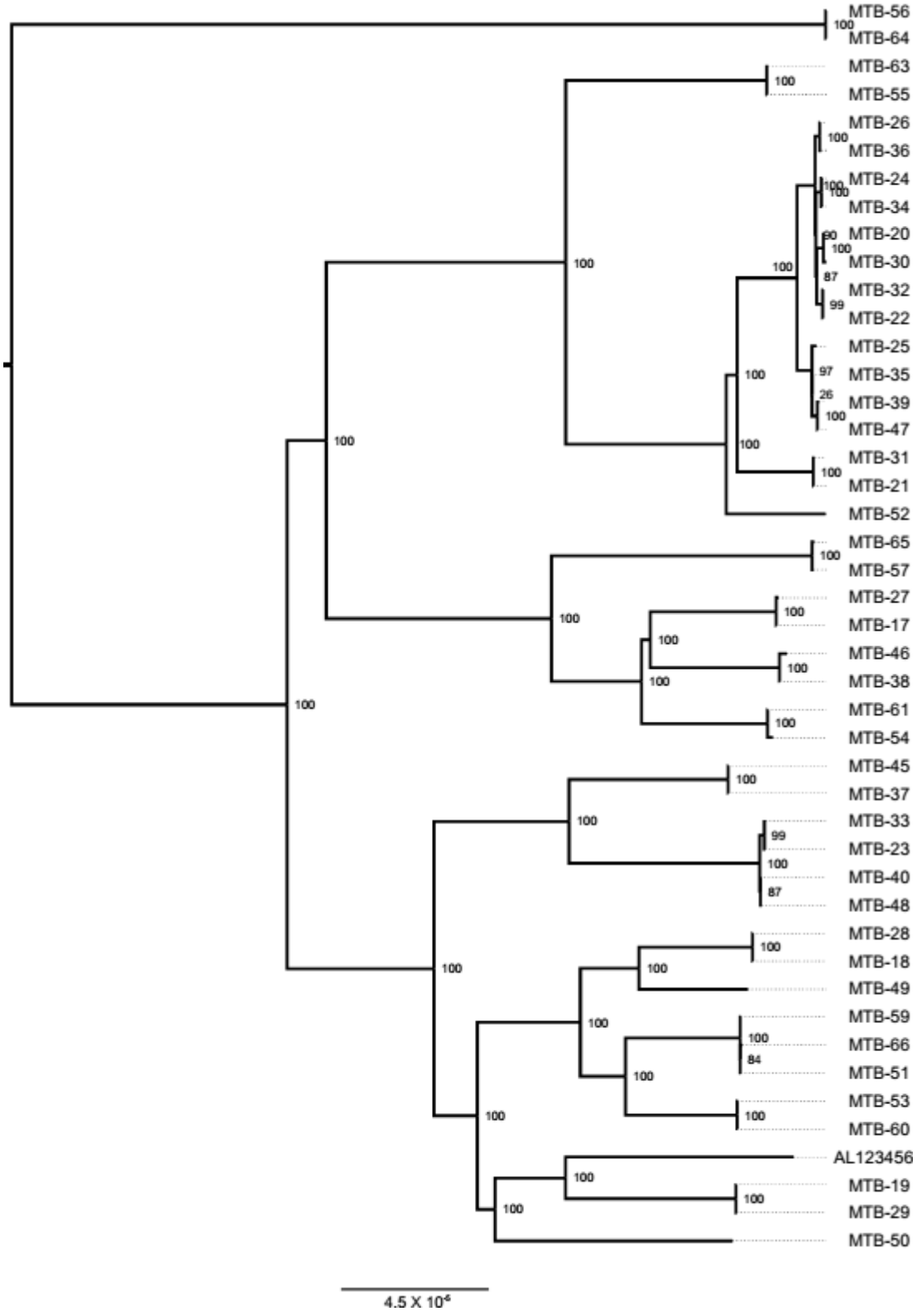
Supplementary Figure 3: Coverage plot of enriched sputum sample MTB-40 mapped against H37Rv. Maximum point on graph is 568.6 and minimum is 0. X axis indicates base position along the genome. Adapted from image generated using Artemis (Carver et. al. 2012 http://www.sanger.ac.uk/resources/software/artemis/).



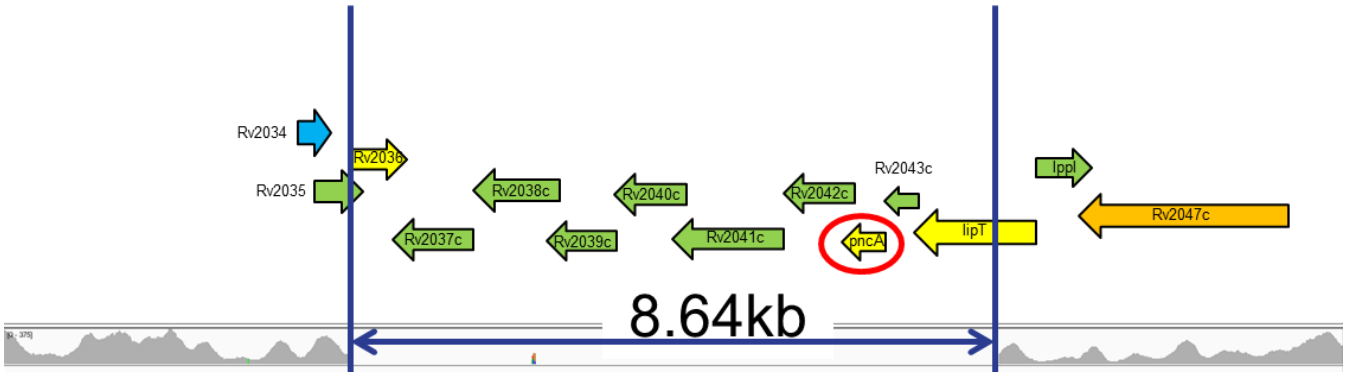
Supplementary Figure 4: Maximum likelihood tree of all samples where at least 1x depth of coverage was obtained. Clade support is indicated by number of bootstrap replicates (out of 100). Scale bar represents substitutions per site. Some samples were identified as being on long branches or positioned close to nodes. Further investigation revealed that they all had at least 10% missing data in the SNP alignment, due to low coverage or heterozygosity, so could not be placed accurately on the tree. The samples indicated in red were removed from the final maximum likelihood tree shown in Supplementary Figure 3.



Supplementary Figure 5: Maximum likelihood tree of 45 samples that had high coverage and could be accurately placed on the tree (see Supplementary Figure 2). Clade support is indicated by number of bootstrap replicates (out of 100). The tree was constructed using RAxML. Scale bar represents substitutions per site.



Supplementary Figure 6: Deletion of pncA and surrounding genes identified in patient 7. Coverage plot is shown in grey.



Supplementary table 2: Resistance genotypes identified in this study that passed all quality criteria and were found at greater than 10% frequency. The position of the mutation identified in column D refers to H37Rv (AL123456.3). Support on the forward, reverse and from individual nucelotides is shown. Any variants that didn't match the observed phenotype were cross-references against the literature, and are discussed in the main text.

Excel spreadsheet