Title: Same-day diagnostic and surveillance data for tuberculosis via whole genome sequencing of direct respiratory samples.

Authors

Antonina A. Votintseva[1#□], Phelim Bradley[2□], Louise Pankhurst[1□], Carlos del Ojo Elias[2], Matthew Loose[3], Kayzad Nilgiriwala[4], Anirvan Chatterjee[4], E. Grace Smith[5,6], Nicolas Sanderson[1], Timothy M. Walker[1], Marcus R. Morgan[7], David H. Wyllie[1,6,8], A. Sarah Walker[1,9], Tim E. A. Peto[1,9], Derrick W. Crook[1,6,8+], Zamin Iqbal[2+#]

Authors' affiliations

[1]Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom

[2]Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, UK

[3]School of Life Sciences, University of Nottingham, Nottingham, NG7 2UH

[4]Foundation for Medical Research, Mumbai

[5]Regional Centre for Mycobacteriology, PHE Public Health Laboratory Birmingham. Heartlands Hospital, Birmingham B9 5SS, UK

[6]Public Health England, Wellington House, 133-155 Waterloo Rd, Lambeth, London SE1 8UG

[7]Microbiology Laboratory, John Radcliffe Hospital, Oxford University Hospitals NHS Trust, Oxford, OX3 9DU, United Kingdom

[8]The Jenner Institute, University of Oxford, Roosevelt Drive, Oxford OX3 7DQ, United Kingdom

[9]National Institute for Health Research (NIHR) Oxford Biomedical Research Centre, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom

+ These authors contributed equally

 These authors contributed equally

[#]Corresponding authors:

Dr Antonina A. Votintseva; Address: Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Level 7, Oxford, OX3 9DU, United Kingdom[1].

Email: a.votintseva@gmail.com

Dr Zamin Iqbal; Address: Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford, OX3 7BN.

Email: zam@well.ox.ac.uk

Running title: Same-day TB WGS from direct respiratory samples

Abstract

Routine full characterization of *Mycobacterium tuberculosis* (TB) is culture-based, taking many weeks. Whole-genome sequencing (WGS) can generate antibiotic susceptibility profiles to inform treatment, augmented with strain information for global surveillance; such data could be transformative if provided at or near point of care.

We demonstrate a low-cost DNA extraction method for TB WGS direct from patient samples. We initially evaluated the method using the Illumina MiSeq sequencer (40 smear-positive respiratory samples, obtained after routine clinical testing, and 27 matched liquid cultures). *M. tuberculosis* was identified in all 39 samples from which DNA was successfully extracted. Sufficient data for antibiotic susceptibility prediction was obtained from 24 (62%) samples; all results were concordant with reference laboratory phenotypes. Phylogenetic placement was concordant between direct and cultured samples. Using an Illumina MiSeq/MiniSeq the workflow from patient sample to results can be completed in 44/16 hours at a reagent cost of £96/£198 per sample.

We then employed a non-specific PCR-based library preparation method for sequencing on an Oxford Nanopore Technologies MinION sequencer. We applied

this to cultured *Mycobacterium bovis* BCG strain (BCG), and to combined culture-negative sputum DNA and BCG DNA. For flowcell version R9.4, the estimated turnaround time from patient to identification of BCG, detection of pyrazinamide resistance, and phylogenetic placement was 7.5 hours, with full susceptibility results 5 hours later. Antibiotic susceptibility predictions were fully concordant. A critical advantage of the MinION is the ability to continue sequencing until sufficient coverage is obtained, providing a potential solution to the problem of variable amounts of *M. tuberculosis* in direct samples.

Introduction

The long-standing gold standard for *Mycobacterium tuberculosis* drug susceptibility testing (DST) is the phenotypic culture-based approach, which is time-consuming and laborious. First-line tuberculosis (TB) treatment includes four drugs (rifampicin, isoniazid, ethambutol and pyrazinamide) but with the spread of multi-drug resistant strains, there is a growing need for data on second-line drugs, including the fluoroquinolones, and aminoglycosides.

Due to long turnaround times for phenotypic testing (up to two months), these are often preceded by WHO-endorsed molecular methods such as the GenoType MTBDRplus and MTBDRsl assays (Hain Lifescience GmbH, Germany), and Xpert MTB/RIF (Cepheid, USA). These potentially culture-free, PCR-based tests rapidly identify species and detect the most common drug resistance conferring mutations. However, this technology is limited by the number of mutations that can be probed. This limitation is of concern, given the many low frequency drug resistance conferring mutations in *M. tuberculosis,* particularly for second-line drugs (1). Consistent with this concern, the proportion of phenotypically resistant samples which are detectable by MTBDRplus range from 21-25% for the second-line drugs capreomycin and kanamycin (2) to 98.4% and 91.4% for the critical first-line drugs rifampicin and isoniazid (3). A potential solution is to sequence amplicons targeting a wider range of resistance conferring genes, as

previously demonstrated (4).

The potential of whole genome sequencing (WGS) as a diagnostic assay has been repeatedly identified (5-7). Recent studies based on WGS of mycobacteria have evaluated WGS-based susceptibility predictions (1, 8-10), species identification, and elucidation of epidemiology (11-16). This has culminated in the first successful application of WGS as a clinical diagnostic for mycobacteria from early positive liquid cultures (16). Moreover, WGS was performed at a cost comparable with existing phenotypic assays and offered faster turnaround times.

Generating WGS information directly from patient samples, and avoiding the need for culture, would be transformative. However, direct samples contain highly variable amounts of mycobacterial cells mixed with other bacterial and human cells; the latter accounting for up to 99.9% of DNA present. Furthermore, mycobacterial cells may aggregate due to the high mucus content of some samples; meaning sample volume and Acid Fast Bacillus (AFB) count may not represent the total quantity of mycobacteria available. Direct samples therefore require pre-processing to homogenize and enrich for mycobacteria by depleting other cells/DNA. The challenges of direct sample processing were illustrated by two studies assessing the feasibility of WGS directly from clinical samples (17-18). By sequencing eight smear-positive sputum samples subjected to differential lysis followed by DNA extraction with a commercial kit, Doughty and colleagues were able to achieve only 0.002-0.7X depth of coverage for *M. tuberculosis* with 20.3-99.3% of sequences mapping to the human genome. 7/8 samples could be assigned to *M. tuberculosis* complex, but none had sufficient

data for drug susceptibility prediction. In a second study, Brown and colleagues applied a SureSelect target enrichment method (Agilent, USA) to capture *M. tuberculosis* DNA prior to WGS. 20/24 smear-positive samples achieved 90% genome coverage with ≥20x depth; sufficient for prediction of species and antibiotic susceptibility. However, the protocol was slow (2-3 days) and may be prohibitively expensive for use in low-income settings.

In this study we test a simple low-cost DNA extraction method using Illumina MiSeq WGS on 40 smear-positive, primary respiratory samples from *M. tuberculosis* infected patients. We evaluate the protocol in terms of DNA obtained, species assignment of the sequenced reads, and our ability to obtain key clinical data (detection of *M. tuberculosis* and antibiotic susceptibility prediction) along with epidemiological information (placement on phylogenetic tree). These data would enable a single test to deliver the core information for both patient and public health in <48 hours using Illumina-based WGS. We also develop an approach for WGS using the highly portable, random-access, Oxford Nanopore Technologies (ONT) MinION, reducing potential turnaround time to below 12 hours.

Results

DNA extraction protocol and evaluation of Illumina sequencing output

DNA was extracted from 40 ZN-positive direct respiratory samples, of which 38 were culture-confirmed *M. tuberculosis* ("culture-positive") and 2 were culture-negative. DNA was also extracted from 28 available corresponding mycobacteria growth indicator tube (MGIT) cultures. All direct samples were the remainder of specimens available after processing by the routine laboratory, and therefore had variable volume (median 1.5 ml, IQR 0.5-3.1, range 0.25-15) and age (median 30 days from collection to processing, IQR 15-45, range 0-67). Most direct samples (78%; 31/40) could therefore be considered suboptimal on the basis of either low volume (≤1ml) or long storage time (≥30 days) or both.

After DNA extraction, 33/40 (83%) direct samples and all 28 MGIT cultures yielded ≥0.2 ng/µl DNA, the amount recommended for MiSeq Illumina library preparation (Figure 1).

There was no evidence that DNA yield was affected (either in multivariable or univariable models) by (1) sample type (sputum or bronchoalveolar lavage) (p=0.94; univariable linear regression), (2) AFB scorings (from +1 to +3) (p=0.56), (3) storage time prior to DNA extraction (days from collection)

198 (p=0.51) and (4) sample volume (p=0.28). Although DNA concentration was
199 measured and recorded after extraction, further data on DNA quality (e.g. DNA
200 Integrity Number (DIN) provided by TapeStation (Agilent, USA)) were not
201 routinely recorded.
202
203 In total 39/40 direct samples with detectable DNA (37 culture-positive, 2
204 culture-negative) and 27/28 MGIT cultures were sequenced on an Illumina
205 MiSeq. One MGIT culture was not sequenced because the corresponding direct
206 sample failed to yield measurable DNA. We used a lower than recommended
207 DNA concentration threshold for MiSeq library preparation (>0.05 ng/μl rather
208 than >0.2 ng/μl) on the basis of previous experience of sequencing mycobacterial
209 cultures with suboptimal amounts of DNA (19). 6/40 (15%) samples yielded
210 DNA below the 0.2 ng/μl threshold. All sequenced direct samples produced ≥1.5
211 million reads (median 3.6 million, IQR 2.9-5.0, range 1.5-12), as did all MGIT
212 cultures (median 3.1 million, IQR 2.8-3.3, range 2.0-4.1).
213
214 **Contamination levels of direct and MGIT samples**
215
216 We assigned reads to categories *M. tuberculosis*, human, naso-pharyngeal flora
217 (NPF) and "other" by mapping (see Methods). 77% (30/39) of direct samples
218 contained <10% human reads. However, only 46% (18/39) contained <10% NPF
219 and other bacterial reads, and 26% (10/39) contained >40% of reads from non-
220 mycobacterial, non-NPF, bacteria (Figure 2a). By comparison, MGIT culture
221 samples showed much less contamination, as expected (Figure 2b).
222

Recovery of *M. tuberculosis* genome

Figure 3a shows the distribution of the *M. tuberculosis* reference genome depth of coverage across direct samples. 21/39 samples have more than 12x depth and recover more than 90% of the genome, and 14/39 samples have <3x depth and recover less than 12% of the genome. The vertical dotted line delineates our threshold of 3x coverage, below which resistance predictions were not made. Figure 3b shows the amount of contamination (reads not mapping to *M. tuberculosis*) per sample. Ten samples had <15% contaminant reads, although contamination levels increased as high as 75% before the proportion of the *M. tuberculosis* genome recovered started to drop. Low numbers of *M. tuberculosis* reads could also reflect poor DNA quality from samples stored for long periods, as most of the samples with <80% reference genome coverage (12/17, 71%) were more than 3 weeks old before extraction.

Concordance of results from direct and MGIT samples

We took a set of 68,695 high quality single-nucleotide polymorphisms (SNPs) obtained from analysis of 3480 samples (1), and genotyped all samples at these positions (see Methods). This allowed us to calculate a genetic distance between the 17 paired MGIT and direct samples (after excluding 10 pairs where the direct sample had <5x coverage, to avoid systematic undercalling in direct samples). The median (and modal) SNP difference was 1 (Figure 4a), with one outlier pair of samples that differed by 1106 SNPS, discussed below, and all other differences ≤22 SNPs.

We placed 17 paired direct and MGIT samples on the phylogeny from (1) (see Methods). Our samples were distributed across global diversity (Figure 4b; tree thinned to aid visibility). For the pair with 1106 SNP differences, the direct sample was isolated on the tree, but the MGIT sample was placed very closely to 3 other pairs (0 SNP difference to one MGIT sample, and 5 SNP differences to the others). Although this might result from different strains being present within the host, a within-laboratory labelling error or cross-contamination is also possible.

No evidence of higher diversity in direct samples

Comparing direct/MGIT pairs where both samples had at least 20x mean depth of coverage on the *M. tuberculosis* reference, the median number of high confidence (see Methods) heterozygous sites was 25 in both direct and MGIT samples. There was no clear trend of greater genome-wide diversity in direct samples (Supplementary Figure 1).

Detection of *M. tuberculosis* in culture positive/negative samples

All sequenced culture-positive (37/39) direct *M. tuberculosis* samples were successfully identified by Mykrobe predictor to complex level (37/37) and 95% to species level (35/37), including 13/37 (35%) where the mean depth of coverage was <3x. All MGIT cultures were identified as *M. tuberculosis*. We were also able to identify *M. tuberculosis* in 2/2 direct samples with low AFB scores

(+1) and no growth in MGIT culture; these may represent dead bacilli from a patient undergoing treatment.

Antibiotic resistance prediction

In total 168 predictions for first-line (n=96) and second-line (n=72) antibiotic susceptibility were made for the 24/37 (65%) direct samples which had at least 3x depth (Supplementary Tables 1,2). For the 13/37 (35%) samples that had <3x depth, no resistance predictions were made. This included 1/2 culture-negative samples.

92/96 (96%) predictions for the first-line antibiotics were concordant with reference laboratory DST. The four mismatches (three pyrazinamide mixed genotypes with both R and S alleles present, and one rifampicin resistant genotype with sensitive phenotype) were found across three samples, all from the same patient (patient 2 in Supplementary Table 2) who had a variable phenotype for rifampicin and pyrazinamide. The resistant genotype for rifampicin was consistent across all three samples from this patient (rpoB_I491F). There is evidence that this mutation causes resistance, but that the phenotype is often reported as sensitive (27,28,1). The mixed genotype for pyrazinamide was again consistent with presence of both R and S alleles on pncA_V7L across all three samples, whereas the phenotype varied. This mutation is also known to confer resistance in samples reported as phenotypically sensitive (1). Further, 1/3 samples from this patient (sample 602112, Supplementary Table 2) contained two additional mutations conferring

298 resistance to isoniazid and pyrazinamide, katG_S315T and pncA_T135P
299 respectively, which were not detected in the previous or following sample. This
300 variation between genotypes from same-patient samples taken over time may
301 represent ongoing evolution, changing population size, and within-patient
302 diversity of *M. tuberculosis* as previously demonstrated by Eldholm *et al* (29). In
303 addition to the above, WGS provided 72 predictions for second-line antibiotics
304 where DST was not attempted.
305
306 The 13/37 samples that yielded insufficient WGS data for resistance prediction
307 had a higher proportion of other bacterial DNA (Figure 3b; median 96%, IQR 38-
308 70%, vs median 12%, IQR 0-67%, in those where resistance prediction was
309 possible, rank-sum p=0.01).
310
311 **Sub-24 hour turnaround time with Illumina MiniSeq**
312
313 Illumina MiniSeq sequencing for three samples (single run; 1 pure BCG, 2
314 negative sputum DNA spiked with BCG DNA) was completed in 6 hours 40
315 minutes. BCG reference genome coverage was 31-33x in spiked samples, and 84x
316 in pure BCG (Table 1). In all cases the species/strain was correctly identified as
317 *M. bovis* strain BCG, and pyrazinamide resistance was correctly identified due to
318 mutation H57D in *pncA.*
319
320 **Modified method for ONT MinION**
321

A new PCR-based rapid 1D MinION protocol was tested using extracted BCG DNA, ZN-negative sputum DNA spiked with BCG DNA, and R9 flowcells (see Methods). Analysis of genome-wide coverage distribution confirmed that use of PCR had not led to significant coverage bias (Supplementary Figure 2), and that >95% of the reference genome attained coverage >5x for all samples. In all cases Mykrobe correctly identified the species/strain as *M. bovis* strain BCG (Table 2). Amplification with Phusion High-Fidelity master mix resulted in the highest yield (760Mb, with 68x coverage of BCG). All MinION experiments resulted in correct identification of the H57D mutation in *pncA* that confers pyrazinamide resistance in BCG, but in the 5% spike this call was filtered out as it only had kmer coverage of 1x on the resistant allele, and did not achieve the required confidence threshold. In all cases, no false resistance calls were made, but deep coverage was needed to be able to genotype all 175 mutations in the catalogue (Supplementary Table 3). Although the pure BCG/R9 and 15% BCG/Phusion/R9 runs missed only 3/175 and 1/175 mutations respectively, (Table 2), only the R9.4 sequencing run (below) allowed all mutations to be typed.

In all 5 samples sequenced on R9 flowcells, data yield was highest at the start of the run, with consistent yield profiles. For the Phusion/15% run we obtained over 65% of the data in 8 hours, and 80% in 10 hours (Supplementary Figure 3). Despite the high sequencing error rate (Supplementary Figure 4), high accuracy genotyping of known SNPs/indels was achievable as described above.

Using two independent methods (see Methods) we measured a strong bias in the distribution of SNP errors in the consensus of the MinION data. Both methods

agreed the bias was systematic, consistent with a strong A->G error bias within a 1D read (Supplementary Tables 4,5), but differed in their determination of the strength of the bias (28% versus 50% A->G respectively). A filter to ensure SNP calls have support from reads mapping to both strands could remove such errors.

12.5 hour turnaround time with ONT R9.4 MinION

We sequenced a single sample (15% BCG spiked ZN-negative sputum) on the latest R9.4 MinION flowcell (see Methods). Yield was 1.3Gb in 48 hours. We were able to detect *M. tuberculosis* complex, identify the strain as BCG, detect the correct pyrazinamide resistance mutation and correctly place the sample on the phylogenetic tree after 1 hour of sequencing. After 3 hours 170/175 mutations were genotyped confidently, after 4 hours we had definitive results for all drugs except streptomycin, and after 6 hours we had definitive results for streptomycin and could stop sequencing. One pyrazinamide mutation remained un-genotyped, but since we already had a confident resistance call for pyrazinamide, there would be no need to continue. Sufficient coverage on the final mutation was obtained after a further 3 hours (9 hours total sequencing; Table 3). Incorporating 6.5 hours for decontamination, DNA extraction and sample preparation (Figure 5), this would give a turnaround time of 7.5 hours for identifying species, phylogenetic placement and initial susceptibility predictions, and 12.5 hours for complete results.

We took our phylogenetic placement of the MiniSeq BCG data as truth, 4 SNPs distant from a BCG sample on the predefined tree. After 1 hour of sequencing with R9.4, we were able to confidently genotype 22694 of the 68695 SNPs, placing the sample at the correct leaf of the tree, at an estimated distance of 3 SNPs. Thus, our genotyping on 1D nanopore reads had at most 7 errors (=3+4) out of 22694 SNPs - an error rate below 0.03%.

Finally, based on the performance of the 1.3Gb R9.4 sequencing run, we estimate (see Methods) that full susceptibility prediction would fail to be generated for 17/39 of the sputum samples sequenced here (MiSeq) with <8% *M. tuberculosis*. However, for the 11/39 samples with >84% *M. tuberculosis*, species-identification and initial susceptibility predictions would be obtained within 20 minutes of sequencing, and full results within 93 minutes (Supplementary figure 8 and Supplementary Table 6).

Costing

Reagent costs (sample decontamination, extraction, sequencing library preparation, and sequencing) per sample were £96 (MiSeq, 12 samples/run), £198 (MiniSeq, 3 samples/run), £515 (R9 MinION, 1 sample/run), £101-172 (R9.4 MinION, between 3 and 5 samples/run, approximate cost as multiplexing kit not yet available). See Supplementary Table 7 for details.

Discussion

Anticipating a growing knowledge-base of the molecular determinants of antibiotic resistance (1), we have developed a method of extracting and purifying mycobacterial DNA from primary clinical samples and producing accurate sequence data in a clinically useful timeframe. We have demonstrated: first, that direct WGS of sputum is possible, and gives genotypic DST predictions which are concordant with phenotype and concordant phylogenetic placement with culture-based sequencing. Second: using an Illumina MiSeq sequencer, we can obtain results within 48 hours for <£100 consumable costs per sample. Using an Illumina MiniSeq can deliver a same-day test (16 hours) for an estimated consumable cost of £198 per sample. Although the costs presented here only represent reagents, they are still likely to be below that of traditional phenotyping (£518 to provide first-/second- line DST and MIRU-VNTR in a bottom-up costing including, for example, consumables, staff time and overheads (16); versus £495 for MiSeq under the same costing model). The MiSeq consumables cost is also well below that of the SureSelect procedure (£203 per sample) (18).

The World Health Organisation (WHO) has called for affordable and accessible point-of-care TB diagnostics, including for DST. Current molecular assays provide partial information on some drugs, but do not easily scale to incorporate a growing list of recognized resistance mutations. Furthermore, additional assays are currently needed where surveillance or outbreak detection are indicated, at additional cost. A single assay providing diagnostic information, and data for surveillance and outbreak detection is therefore an attractive prospect.

In cities where there are large numbers of TB cases (for example upward of 65,000 TB cases per years in Mumbai) centralized sequencing services taking advantage of high throughput Illumina sequencing platforms may be applicable. However, at current prices in 2017, the relatively high capital costs, and requirement for a well-equipped laboratory are an impediment to implementation across the full range of locations across the world. For a complete solution, the ability to function in varied low-tech environments is a practical necessity. The MinION can deliver this at least on a small-scale, as demonstrated in Guinea last year during the Ebola outbreak (30). We confirm here that, despite the high error rate in reads, given deep coverage, it is possible to accurately genotype resistance SNPs using the MinION method applied here. However, widespread implementation would require much larger feasibility studies, similar to those recently conducted to implement MiSeq sequencing from MGIT samples for *Mycobacteria* diagnosis by Public Health England.

Since with Illumina technology the depth of sequencing is determined in advance (by the number of isolates run in a batch), the small amount of *M. tuberculosis* in a direct sample can result in test failures. In this experiment *M. tuberculosis* identification and susceptibility prediction failed in 2/39 and 13/37 samples respectively. MinION sequencing in theory allows sequencing to continue until sufficient coverage has been obtained, giving faster results when there is high load, and avoiding this type of failure when the load is low. The throughput obtained here with a 15% BCG-spiked sputum sample and R9.4 flowcells (1.3 Gb) would allow a turnaround time of 12.5 hours (sample to complete results);

or only 7.5 hours to detection of *M. tuberculosis*, pyrazinamide resistance and placement on a phylogenetic tree.

We have predicted that species and initial DST could be generated after 20 minutes (mean) of sequencing using MinION R9.4, and final DST within 150 minutes; providing the *M. tuberculosis* concentrations are sufficient (>=20% of total sequencing reads in this study). For these samples, it would be possible to multiplex sequencing and reduce per-sample costs. Conversely, for samples with low *M. tuberculosis* concentrations nanopore sequencing would not provide sufficient data within 48 hours. Although these predictions are based on a single R9.4 sequencing run, the data demonstrate clear scope for technology-driven improvement, either through improved mycobacterial enrichment and/or non-mycobacterial DNA depletion (Figure 2a), higher sequencing yield, or real-time filtering of contamination (31).

Were this methodology implemented in clinical practice, we would expect a portion of direct sample would be retained for culture in all cases; this would be available for resequencing in case of insufficient sequencing depth, and would allow distinction between live and dead bacilli. Unlike our study, where we had to use sample discards after clinical processing, if implemented, increased sample volume could be used for sequencing, closer to the time the sample was taken, presumably with greater success. Both Illumina and ONT technologies would require some level of sample batching, but for both, turnaround time is likely to be much faster than traditional phenotyping and could challenge same-day molecular tests such as Xpert MTB/RIF.

In conclusion, diagnostic and surveillance information direct from patient specimens can now be obtained in 16/44 hours on Illumina MiniSeq/MiSeq platforms, a considerable step forward. In addition, the ONT sequencing platform may offer the same information in as little as 7-12.5 hours. Faster and more automated sample processing, as well as cost reductions, are clearly needed for adoption in low-income settings. Achieving this would revolutionize the management of TB.

Materials and Methods

Sample selection and processing

Direct respiratory Ziehl-Neelsen (ZN)-positive samples with acid-fast bacilli (AFB) scorings from +1 to +3 used in this study had been originally collected from patients with subsequently confirmed *M. tuberculosis* infections at the John Radcliffe Hospital, Oxford Universities NHS Foundation Trust, Oxford, UK (n=18), and Birmingham Heartlands Hospital NHS Foundation Trust, Birmingham, UK (n=22). 2/18 Oxford samples were culture negative specimens taken 2.5 months apart from the same patient undergoing treatment for *M. tuberculosis*. If available, corresponding Mycobacterial Growth Indicator Tube (MGIT) cultures

were collected for each direct sample (Oxford n=11, Birmingham n=17). Two ZN and culture negative direct respiratory samples were also collected from the John Radcliffe Hospital.

The discarded direct samples were collected only after sufficient material had been obtained for the routine diagnostic workflow, including the requirement to ensure that enough sample volume remained if re-culture was requested. Consequently, study samples were of lower volume and quality than would be the case if the method were used routinely. While waiting for the routine laboratory results, samples were stored at +4C and later processed in batches of 5-12. All ZN-positive samples were digested and decontaminated with NAC-PAC RED kit (AlphaTec, USA). Direct samples and corresponding MGIT culture aliquots (1 mL) were heat inactivated in a thermal block after sonication (20 min, 35 kHz) for 30 min and 2 h at 95C, respectively. MGITs were inactivated for 2 hours owing to their high bacterial load. Before DNA extraction samples were stored at +4C.

DNA extraction and Illumina MiSeq sequencing

Mycobacterial DNA from MGIT cultures was extracted using a previously validated ethanol precipitation method (19). DNA from ZN-positive direct samples was extracted using a modified version of this protocol. These modifications included a saline wash followed by MolYsis Basic5 kit (Molzym, Germany) treatment for the removal of human DNA, and addition of GlycoBlue co-precipitant (LifeTechnologies, USA) to the ethanol precipitation step (Supplementary Figure 5).

Libraries were prepared for the MiSeq Illumina sequencing using a modified Illumina Nextera XT protocol (19). Samples were sequenced using the MiSeq Reagent Kit v2, 2 x 150bp in batches of 9-12 per flow-cell. Median library size (Tapestation, Agilent, USA) was 627 bp (IQR 495 – 681). Median reads available per sample was 3.2 million (IQR 2.8 – 4.1 million); this would yield a median depth of approximately 213, given pure *M. tuberculosis,* although in this study we anticipated non-mycobacterial reads would be present.

DNA extraction for ONT MinION and Illumina MiniSeq sequencing

ZN/culture-negative sputum and BCG (Pasteur strain; cultivated at 37C in MGIT tubes) DNA was extracted using a modified version of that in (19). Briefly, following a saline wash, samples were re-suspended in 100 µL of molecular grade water and subjected to three rounds of bead-beating at 6 m/s for 40 seconds. The beads were pelleted by centrifugation at 16,100 xg for 10 minutes and 50 µL supernatant cleaned using 1.8x volume AMPure beads (Beckman Coulter, UK). Samples were eluted in 25 µL molecular grade water, and quantified using the Qubit fluorimeter (Thermo Fisher Scientific, USA). (Steps I, III, V and VI of Miseq protocol, Supplementary Figure 5.)

MiniSeq sequencing

Extracted ZN-negative sputum DNA and pure BCG DNA were combined in a 50:50 ratio (0.5 ng each) and libraries prepared alongside pure BCG DNA (1 ng) using a modified Illumina Nextera XT protocol (19). BCG and two BCG+sputum

DNA samples were sequenced at Illumina Cambridge Ltd. UK, using a Mid Output kit (FC-420-1004) reading 15 tiles and with 101 cycles.

MinION sequencing

All MinION sequencing utilized the best available sample preparation kit for our samples and flow cells (R9/R9.4 flowcells and PCR-based sample preparation, as described below). A single ZN-negative sputum extract was divided into three equal concentration aliquots (187 ng), and BCG DNA added at 5%, 10% and 15% of the total sputum DNA concentration. These 5-15% spikes represent the lower end of the spectrum seen in the MiSeq samples above (see Figure 2a). These samples, along with pure BCG DNA, were prepared following ONTs PCR-based protocol for low-input libraries (DP006_revB_14Aug2015), using modified primers supplied by ONT, a 20 ng DNA input into the PCR reaction, and LongAmp *Taq* 2X Master Mix (New England Biolabs, USA). PCR conditions were as follows: initial denaturation at 95°C for 3 minutes, followed by 18 cycles of 95°C for 15s, 62°C for 15s, and 65°C for 2.5 minutes, and a final extension at 65°C for 5 minutes. Samples were cleaned in 0.4x volume AMPure beads and the PCR product assessed using the Qubit fluorimeter and TapeStation (Agilent, UK). The final elution was into 10 µL 50 mM NaCl, 10 mM Tris.HCl pH8.0. Finally, 1 µL of PCR-Rapid Adapter (PCR-RAD; supplied by ONT) was added and samples incubated for 5 minutes at room temperature to generate pre-sequencing mix. The pre-sequencing mix was prepared for loading onto flow cells following standard ONT protocols, with a loading concentration of 50 – 100 fmol.

Using the 15% BCG spiked sputum DNA prepared above, amplification was repeated using Phusion High-Fidelity PCR Master Mix with DMSO (New England BioLabs, USA). Gradient PCR was performed to identify the optimal annealing temperature for recovery of BCG DNA (data not shown). Final PCR conditions were as follows: initial denaturation at 98°C for 30s, followed by 18 cycles of 98°C for 10s, 59°C for 15s, and 72°C for 1.5 minutes, and a final extension of 72°C for 10 minutes. Following PCR, the sample was prepared for sequencing as described above. The final loading concentration was approximately 27 fmol.

The above samples were sequenced using R9 spot-on generation flow cells and the 48-hour protocol for FLO-MIN105 (ONT, UK). Base calling was performed via the Metrichor EPI2ME service (ONT, UK) using the 1D RNN for SQK-RAD001 v1.107 workflow.

Subsequently, a new 15% BCG spiked sputum was prepared as described above using Phusion Master Mix with DMSO. Sequencing was performed using R9.4 spot-on generation flow cells and the 48-hour FLO-MIN106 protocol (ONT, UK). Final loading concentration was 43 fmol. Base calling was performed after sequencing was complete using Albacore (ONT, UK), as base calling via Metrichor failed. Subsequent tests on other samples (data not shown) showed that base-calling could have been performed in real-time – during the run.

Bioinformatic analysis of Illumina data

To determine levels of contamination and *M. tuberculosis* in samples, reads were immediately mapped using bwa_mem (20) to the human reference genome

GRCh37 (hg19) and human reads counted and permanently discarded. Remaining stored reads were then mapped to the *M. tuberculosis* H37Rv reference strain (GenBank NC_018143.2), and any unmapped reads were then mapped to nasal, oral and mouth flora available in the NIH Human Microbiome Project database (http://www.hmpdacc.org/).

Mycobacterial species and antibiotic resistance to isoniazid, rifampicin, ethambutol, pyrazinamide, streptomycin, aminoglycosides (including capreomycin, amikacin and kanamycin) and fluoroquinolones (including moxifloxacin, ofloxacin, and ciprofloxacin) was predicted using Mykrobe predictor software (21) v0.3.5, updated with a new validated catalogue of resistance conferring genetic mutations (Supplementary Table 3, from (1)). For samples where the estimated depth of kmer-coverage of *M. tuberculosis* reported by Mykrobe predictor fell below 3x, no resistance predictions were made. The precise command used was:,``mykrobe predict SAMPLE_ID tb -1 FASTQ –panel walker-2015 –min-depth 3".

Phylogenetic analysis of pairs

Conservative SNP calls were made using Cortex (22) (independent workflow, k=31) on 3480 samples from (1). Singleton variants were discarded, and a de-duplicated list of 68695 SNPs was constructed. All samples (from our study and from (1)) were genotyped at these sites using the Mykrobe predictor genotyping model (21). All 27 of the MGIT samples had high coverage, but several of the direct samples had low coverage (Figure 3a, bottom left). For this comparison we excluded pairs where the direct sample had <5x coverage to ensure like-with-

like analysis, leaving 17 pairs. We then measured the number of SNP differences between the paired direct and MGIT samples, counting only sites where both genotypes had high confidence in our Illumina model (difference between log likelihood of called genotype (eg ALT allele) and of uncalled genotype (eg REF allele) greater than 1), and neither site was called as heterozygous.

Samples were placed on the phylogenetic tree of 3480 samples from (1) by identifying the leaf with the fewest SNP differences, across the 68695 sites. Placement therefore returns a closest leaf, and a SNP distance to that leaf.

Statistical analysis

Univariable and multivariable linear regression was used to identify independent factors affecting log10 DNA concentration after extraction. Analyses were performed using Stata 14.1 (2015, StataCorp, USA).

Bioinformatic Analysis of MinION Data

Mykrobe predictor version v0.5.0-6-g6b19d83 was used to predict resistance from the MinION basecalled reads (command: mykrobe predict SAMPLE_ID tb -1 FASTQ –panel walker-2015). This uses an ONT-specific genotyping model, a modification of that published in (21) – specifically it uses a Poisson model of total kmer counts on alternate alleles (instead of using the median kmer coverage), and applies a "genotype confidence" threshold of 100 (difference between log likelihood of called genotype (eg ALT allele) and of uncalled genotype (eg REF allele) greater than 100). Supplementary Figures 6,7 show the genotype confidence distribution split by whether the genotype is correct or not.

Yield and timing were analyzed using Poretools (23). For the R9.4 sample, Mykrobe predictor was applied to the cumulative read output at each hour. Yield of BCG was measured by mapping to a BCG reference (accession BX248333.1).

Phylogenetic placement of the 15% spike BCG sample sequenced on MinION R9.4 was achieved as for the Illumina data - by genotyping 68695 SNPs, and choosing the leaf with the fewest SNP differences across those sites.

MinION error analysis

Error bias in the consensus of MinION R9 1D pure BCG reads was measured in two ways, using reads from the pure BCG sequencing run described above.

1. Reads were mapped to the *M. tuberculosis* reference genome using bwa_mem, and then this was passed to the consensus tool racon (24). The output of this was compared with the BCG reference genome using MUMMER (25). Since we were comparing *M. bovis* BCG strain with its own reference genome, any observed SNPs were either due to sequencing errors, or to evolution since the reference genome was constructed. We assumed the latter were negligible in comparison with the error rate in nanopore reads (Supplementary Figure 4), and considered all SNPs to be errors. Bias in these errors was observed by looking at isolated SNPs (avoiding alignment artefacts due to nearby indels). The results are shown in Supplementary Table 4.

2. A *de novo* assembly was performed with Canu (26), and then this was compared with the BCG reference genome using MUMMER, as above. Results are shown in Supplementary Table 5.

The mapping approach ((1) above) found 28% of consensus errors were A->G and 60% were T->C. (Note these refer to the SNP with respect to the reference, not to errors within a single read passing through a pore.) The de novo assembly approach found 50% of consensus errors were A->G, and 44% were T->C. Although the estimates differed quantitatively, they agreed on the existence and direction of the bias.

MinION turnaround estimates using empirical *M. tuberculosis* read proportion data

The proportion of *M. tuberculosis* reads found in our clinical samples varied over a considerable range (Figure 2a, blue), with between 0.3% and 97.9% of sequenced DNA coming from *M. tuberculosis*. To model how this distribution might translate into MinION performance, we used hourly timestamps on the R9.4 MinION total DNA yield curve (Supplementary Figure 3), and coverage needed to detect *M. tuberculosis*, pyrazinamide resistance and full susceptibility results, to estimate the turnaround times for all Illumina-sequenced samples, supposing they all were to yield 1.3Gb of MinION reads with the same proportion of reads from *M. tuberculosis* as seen in Figure 2a. The results are displayed in Supplementary Figure 8 and Supplementary Table 6, with samples ordered by increasing proportion of *M. tuberculosis*.

690

691 **Costing analysis**

692 Basic costing included reagents required for sample decontamination, DNA
693 extraction, MiSeq and Nanopore library preparations, and sequencing; correct as
694 of November 2016. Generic laboratory consumables (e.g. pipette tips, tubes)
695 were not included. SureSelect (Agilent, UK) costs, as used by Brown *et al.* (18),
696 were obtained via a company representative and were correct of June 2016.
697 United States Dollars (USD) were converted to Great British Pounds (GBP) at
698 $1.25 USD per GBP. See Supplementary Table 7 for details.

699

700 **Ethics**

701 For this study no ethical review was required because it was a laboratory
702 methods development study focusing on bacterial DNA extracted from discarded
703 samples identified only by laboratory numbers with no personal or clinical data.
704 Sequencing reads identified as human based on fast mapping with BWA were
705 counted and immediately permanently discarded (i.e. never stored
706 electronically).

707

708 **Accession numbers**

709 The MiSeq, MiniSeq and MinION data have been deposited in the Sequence Read
710 Archive (SRA), NCBI, under the study accession number SRP093599.

711

712 **Acknowledgements**

713 We thank Phuong Quan for assistance with statistical analysis, Rachel Norris for
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Table 1: Yield from pure BCG, and from negative sputum spiked with BCG – sequenced on Illumina MiniSeq

	estimated Fmol loaded	Yield (Mb)	Read length (bp)	BCG reference coverage
Pure BCG TB1_N716	800	381	101	84.0
50 % BCG TB1_N718	800	244	101	31.0
50% BCG TB1_N719	800	257	101	33.0

Table 2: Yield from pure BCG, and from negative sputum spiked with BCG - both sequenced with MinION 1D protocol

Model	Sample	Fmol loaded	Read count	Yield/ Mb	Avg read length (kb)	BCG covg depth	H57D R-allele kmer covg+	% mutations typed (# not typed)
R9	Pure cultured BCG	++	297,239	360	1.2	80	17	99 (1)
R9	5% BCG LongAmp	82	182,670	559	2.0	19	1*	47 (93)
R9	10% BCG LongAmp	76	180,507	467	1.8	10	3	56 (77)
R9	15% BCG LongAmp	51	203,285	627	2.0	35	3	90 (18)
R9	15%	27	184,89	758	2.4	68	10	98 (3)

	BCG Phusion		5					
R9.4	15% BCG Phusion	43	754,338	1306	1.7	147	16	100 (0)

914

915 + kmer coverage on resistance allele of the H57D mutation in pncA, known to be
916 present in BCG.
917 ++ data not available
918 * resistance SNP detected but failed confidence threshold, and filtered out
919

920

921 **Table 3: Susceptibility prediction at timestamps during R9.4 run**

922

Hour	%AMR mutations typed	Number of mutations un-genotyped (total 175)	Un-genotyped mutations	Drugs awaiting results
1	57.1	75	*	All except pyrazinamide
2	88.5	20	katG (S700, L141, V633, W191, D142, L704) gid (L26, V41, G34, R47, G117, A205, R118, Q125) rpoB (H445) embB (D328, G406) rpsL (K43) pncA (T47, K48)**	Isoniazid, streptomycin, rifampicin, ethambutol
3	97.1	5	embB (D328) gid (G34, A205) katG (W191) pncA (T47)	Ethambutol, streptomycin, isoniazid
4	98.2	3	gid (G34, A205)	Streptomycin

			pncA (T47)	
5	98.8	2	gid (G34) pncA (T47)	Streptomycin
6-9	99.4	1	pncA (T47)	-
9	100	0	-	-

923

924 * We omit list of un-genotyped mutations here; 75 is too many to list.

925 ** further un-genotyped pncA mutations could be ignored, as H57D had already

926 been detected at 1 hour – sample already predicted to be pyrazinamide resistant.

927 Thus pyrazinamide not listed in column 5.

928

929

930

Figure Legends

Figure 1: DNA extracted (ng/ml) from MGIT cultures and direct clinical samples. Each dot represents a single extraction. Horizontal line at 0.2 ng/µl represents the DNA concentration theoretically required for MiSeq library preparation. Horizontal line at 0.05 ng/µl represents minimum DNA concentration used for MiSeq library preparation from direct samples in this study. One sample not shown as DNA was below detection limits.

Figure 2: Proportion of reads assigned to various species categories in each sample sorted by increasing total count of MTBC reads. **a**) Direct samples show removal of human DNA (red) has been broadly successful, but removal of naso-pharyngeal flora (NPF, green) and other bacteria (purple) had more variable success. **b**) MGIT samples show much more uniform dominance of *M. tuberculosis* reads, as expected after 2 weeks of culture designed to favor mycobacterial growth.

Figure 3: Recovery of *M. tuberculosis* genome in direct samples and robustness to contamination. **a**) Depth versus proportion of the *M. tuberculosis* reference recovered (at >5x depth). Vertical dotted line at 3x depth is threshold used for resistance prediction in this study. **b**) Proportion of contamination (reads not mapping to *M. tuberculosis* reference) versus proportion of genome recovered. Samples with less than 95% of the *M. tuberculosis* genome recovered all have >75% contaminated reads.

Figure 4: Genotypic concordance between direct and paired MGIT samples. **a**) Histogram of genetic (SNP) differences, excluding the one pair which differ by 1106 SNPs; median (and modal) difference is 1 – thus direct sequencing is identifying the same strain of *M. tuberculosis* as culture-based sequencing would. **b**) Placing direct/MGIT pairs on a phylogenetic tree of 3480 samples shows distribution of samples across world diversity. Circle indicates the sequence from the direct sample and cross the sequence from the corresponding MGIT sample; for the 1 pair (of 17) with 1106 differences (turquoise), the MGIT sample places very close to other samples (0 SNP differences to one (MGIT) sample, 5 SNP differences to others), and so is possibly due to a labeling error.

Figure 5: Timelines and cost. We compare the method of Brown et al with the results of this study, using the Illumina MiSeq and MiniSeq, and the ONT MinION. We assume that no step of the process can be initiated after 6pm or before 8am. The method of Brown et al has a rapid extraction step, but also a 20 hour overnight enrichment step, resulting in a 50 hour turn-around time. The DNA extraction process was updated for the MiniSeq and MinION experiments, removing the ethanol precipitation step. In normal use this would take 3 hours. The 1.5 hour orange rectangle on the MinION timelines includes both PCR and the 10 minute sample preparation step. In this experiment, since we used spiked BCG DNA in sputum, we did not use a human depletion step, thus taking only 2 hours. This figure is intended to show comparable real-use timelines, and so the MiniSeq/MinION timelines are shown with 3 hour extraction steps. The MiniSeq

enables a 16-hour turnaround time, by sequencing for only 7 hours. The R9 MinION also delivers sub-24 hour results, but requires one flow-cell per sample. The R9.4 MinION gives a 12.5 hour turnaround time (6 hours of sequencing with real-time (i.e. simultaneous) basecalling when used on a single sample).

Figure 1

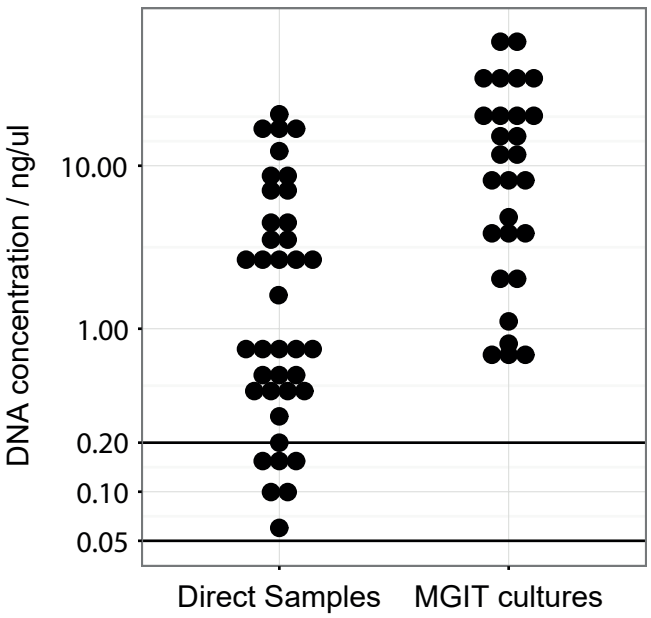

DNA concentration / ng/ul
10.00
1.00
0.20
0.10
0.05
Direct Samples
MGIT cultures

Figure 2

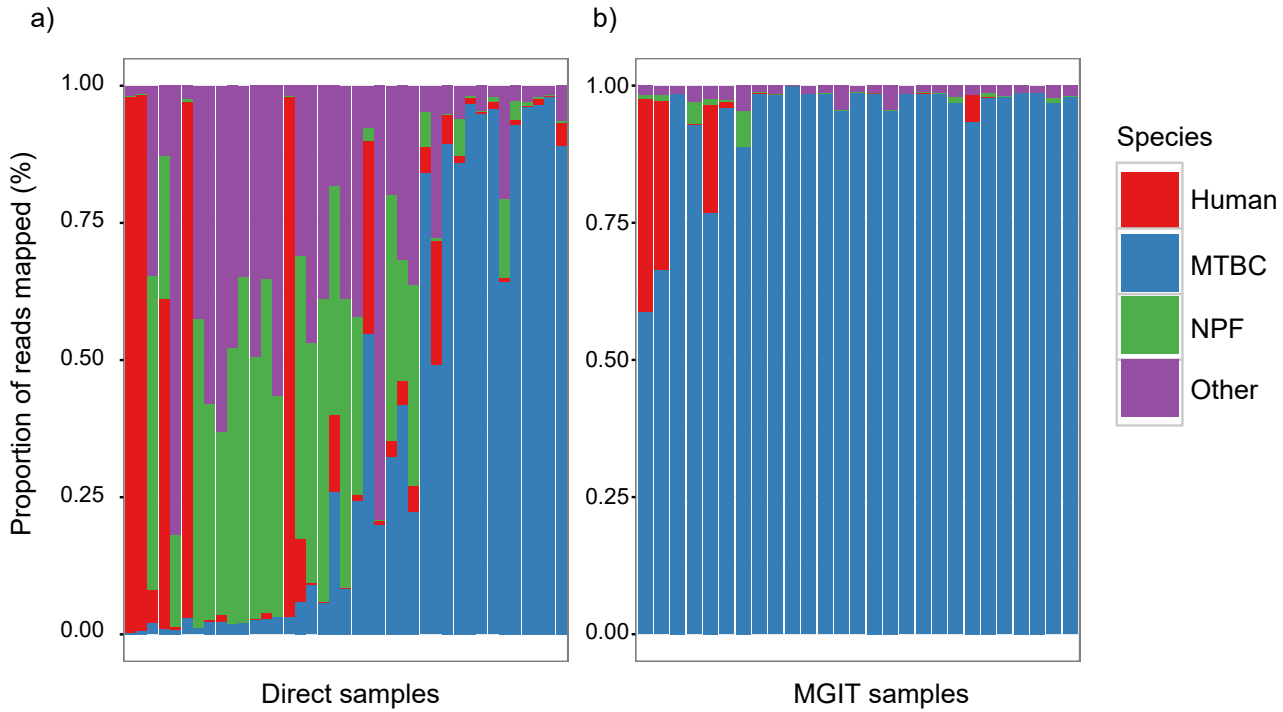

a)
b)
Proportion of reads mapped (%)
1.00
0.75
0.50
0.25
0.00
Direct samples
MGIT samples
Species
Human
MTBC
NPF
Other

Figure 3

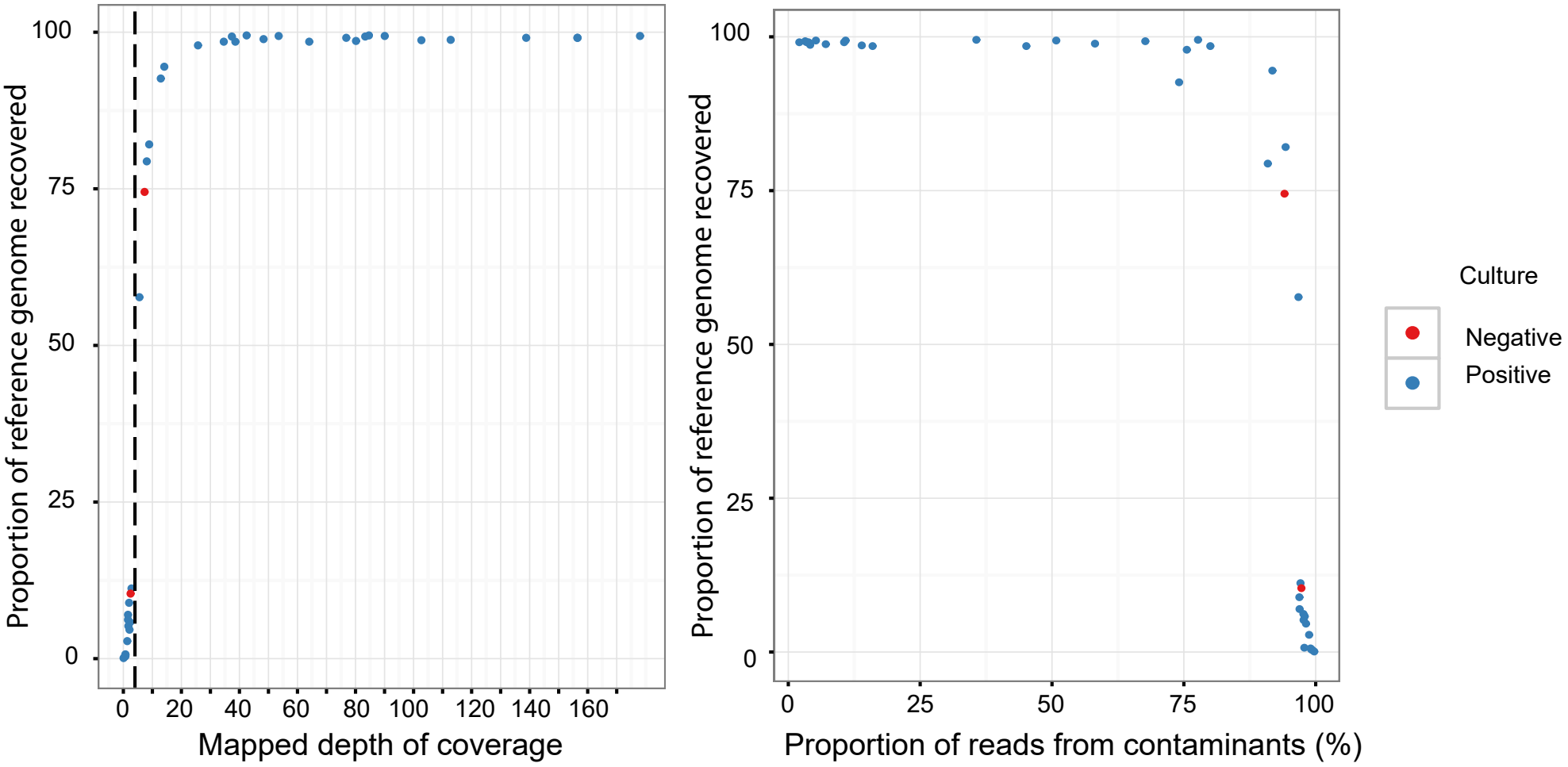
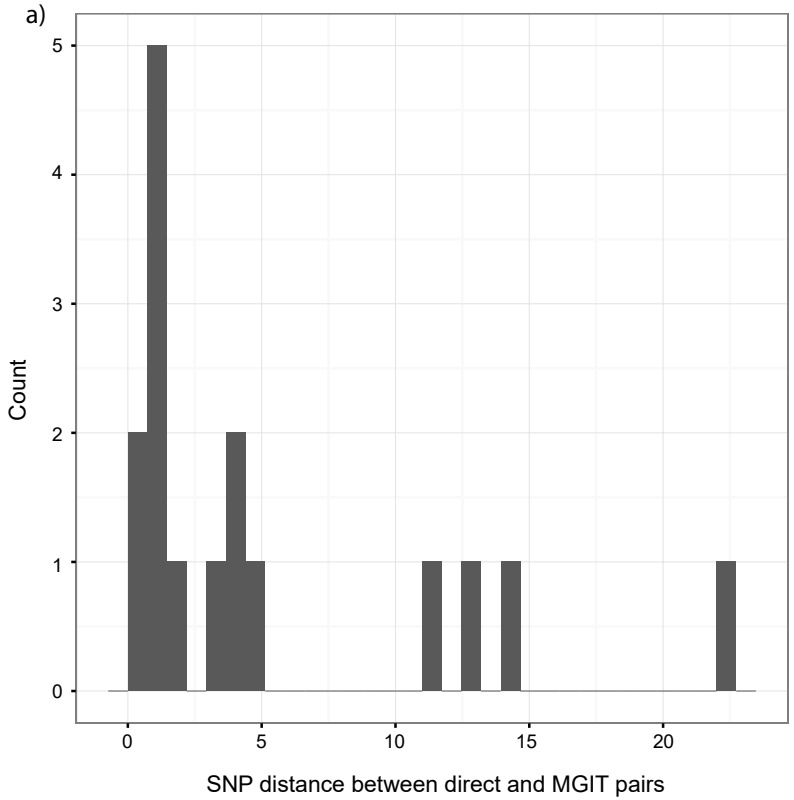

Proportion of reference genome recovered
100
75
50
25
0
0
20
40
60
80
100
120
140
160
Mapped depth of coverage
Proportion of reference genome recovered
100
75
50
25
0
0
25
50
75
100
Proportion of reads from contaminants (%)
Culture
Negative
Positive

Figure 4


a)
Count
0
1
2
3
4
5
0
5
10
15
20
SNP distance between direct and MGIT pairs

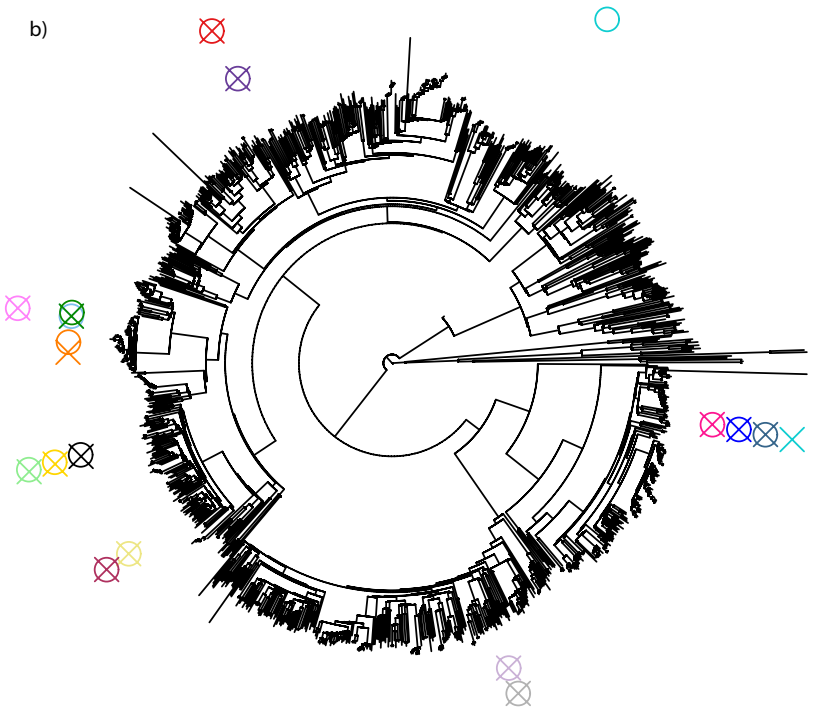
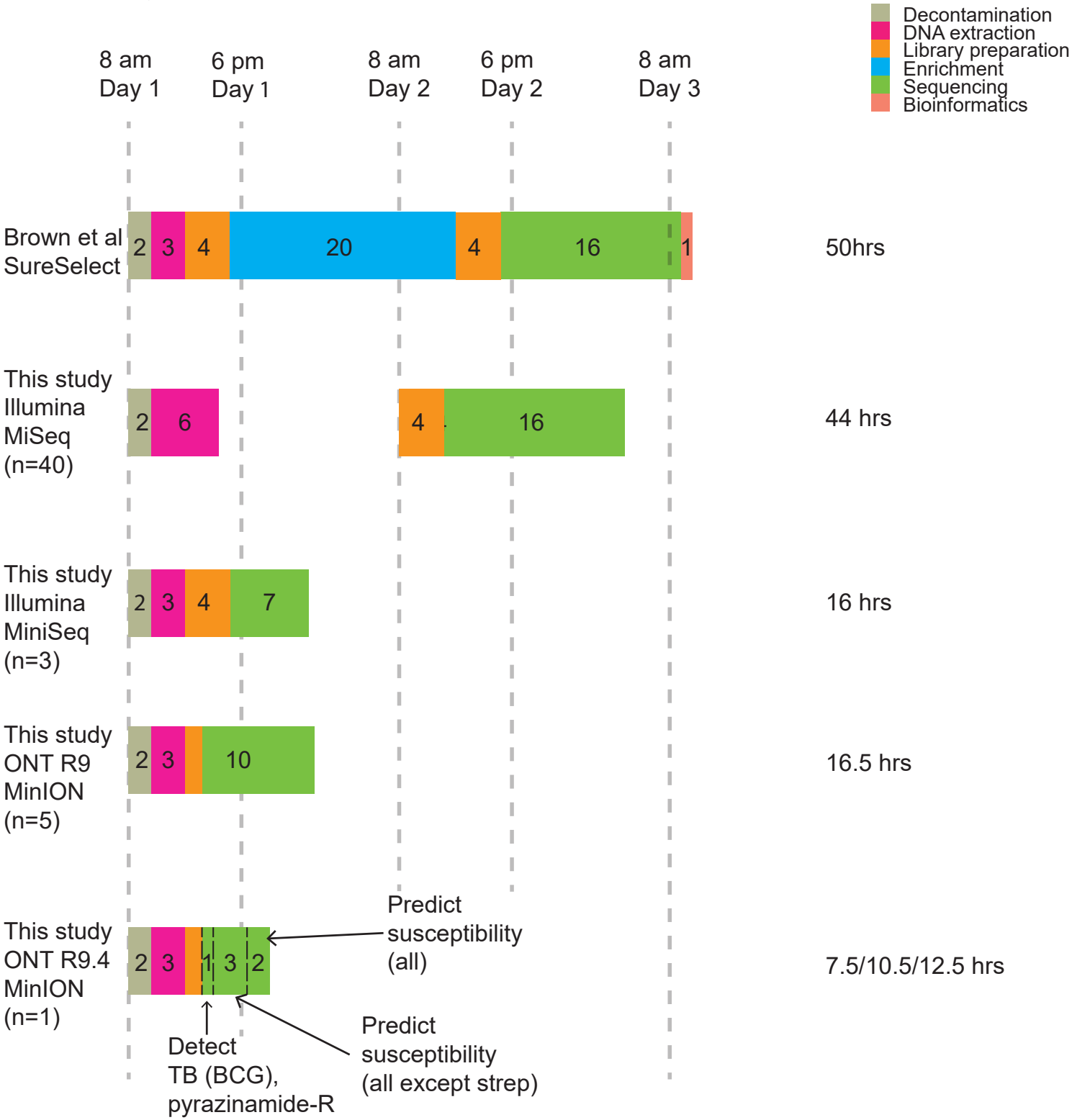

b)

Figure 5


Decontamination
DNA extraction
Library preparation
Enrichment
Sequencing
Bioinformatics
8 am Day 1
6 pm Day 1
8 am Day 2
6 pm Day 2
8 am Day 3
Brown et al SureSelect
2
3
4
20
4
16
1
50hrs
This study Illumina MiSeq (n=40)
2
6
4
16
44 hrs
This study Illumina MiniSeq (n=3)
2
3
4
7
16 hrs
This study ONT R9 MinION (n=5)
2
3
10
16.5 hrs
This study ONT R9.4 MinION (n=1)
2
3
1
3
2
Predict susceptibility (all)
Predict susceptibility (all except strep)
Detect TB (BCG), pyrazinamide-R
7.5/10.5/12.5 hrs