Bringing TB genomics to the clinic: A comprehensive pipeline to predict antimicrobial susceptibility from genomic data, validated and accredited to ISO standards.

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Abstract

Background: Whole genome sequencing shows promise to improve the clinical management of tuberculosis, but bioinformatic tools tailored for clinical reporting and suitable for accreditation to ISO standards are currently lacking.

Methods: We developed tbtAMR, a comprehensive pipeline for analysis of *Mycobacterium tuberculosis* genomic data, including inference of phenotypic susceptibility and lineage calling from both solid and broth (MGIT) cultures. We used local and publicly-availably real-world data (phenotype and genotype) and synthetic genomic data to determine the appropriate quality control metrics and extensively validate the pipeline for clinical use. We combined and curated the large global databases of resistance mutations, fine-tuned for clinical purposes, by minimising false-positives whilst maintaining accuracy.

Findings: tbtAMR accurately predicted lineages and phenotypic susceptibility for first- and second-line drugs, including from broth (MGIT) cultures. We designed and implemented a reporting template suitable for clinical and public health users and accredited the pipeline to ISO standards.

Interpretation: The tbtAMR pipeline is accurate and fit-for-purpose for clinical and public health uses. Report templates, validation methods and datasets are provided here to offer a pathway for laboratories to adopt and seek their own accreditation for this critical test, to improve the management of tuberculosis globally.

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48 # Introduction

49 *Mycobacterium tuberculosis* (Mtb) is the causative agent of tuberculosis (TB), a disease that is globally
50 widespread predominantly affecting people in low- and middle-income communities and is the leading
51 cause of death from a single infectious agent.[1]

52 Treatment of TB infections is lengthy and expensive, requiring combination therapy with more than
53 one antimycobacterial drug prescribed for a minimum of six months for uncomplicated disease. The
54 slow growth of Mtb *in vitro* means that clinicians treating patients with TB must choose treatment
55 regimens before phenotypic drug susceptibility test (DST) results are available, which can take at least
56 two months[2] in some settings. Treatment is usually commenced with four antimycobacterial drugs
57 ('first-line agents'): rifampicin, isoniazid, pyrazinamide and ethambutol, followed by rationalisation to
58 two agents (rifampicin and isoniazid, if susceptible), or an alternative regimen including second-line
59 agents if resistant. Whole genome sequencing (WGS) has the advantage of providing comprehensive
60 results from a Mtb sample in a single test, potentially significantly reducing turnaround time to DST for
61 both first-line and other antimycobacterial drugs, especially when undertaken from Mycobacteria
62 Growth Indicator Tube (MGIT) culture. In addition, WGS can provide increased reliability of resistance
63 detection for some drugs, due to known challenges with phenotypic reproducibility, such as
64 ethambutol and pyrazinamide[3]. WGS data is also being used to provide support for outbreak
65 investigation and contact tracing. Therefore, the use of routine WGS for Mtb has the potential to
66 improve patient management in a clinical setting and public health outcomes, and new Australian
67 national guidelines recommend routine use and reporting of findings for these reasons[4].

68 There are many high-quality bioinformatics tools available for identification of genomic determinants
69 of AMR in Mtb, including Mykrobe,[5] TB-Profiler[6] and ariba[7]. Additionally, databases of genomic
70 determinants implicated in AMR in Mtb have become more comprehensive in recent years, including
71 the WHO Mtb Mutation Catalogue[5,8]. However, they are not designed for reporting within a clinical
72 and public health laboratory (CPHL), often being over-inclusive. This over-inclusivity is useful in
73 research settings but is not suited to a CPHL environment, as high false-positive rates jeopardise clinical
74 care by ruling out effective first-line therapies. Despite the urgent need for routine identification of
75 Mtb AMR from genomic data to inform therapy and the wealth of available bioinformatic resources,
76 few public health laboratories globally have such processes in place to report AMR from WGS data for
77 Mtb. Many factors need to be considered when implementing such an AMR detection program,
78 particularly where patient management may be impacted, including performance of tools in predicting
79 drug-resistance, the impact of laboratory methodologies and existing processes, reporting structure,

80 data management in a CPHL, and test accreditation by national accreditation body to International
81 Organization for Standardization (ISO) standards (ISO15189:2022).[9]

82 Here we demonstrate the validation of a real-time routine analysis pipeline for inference of phenotypic
83 resistance in Mtb, based on detection of genomic variation. We have subsequently gained
84 accreditation to ISO standard 15189:2022 for this pipeline, tbtAMR, for clinical and public-health
85 reporting of genomic AMR detection in Mtb. The pipeline and validation approach are made available
86 here for other laboratories globally to aid in the transition to WGS for genomic AMR reporting.

87

Methods

Implementation

tbtAMR software development

Numerous software options exist for interrogation of Mtb for the detection of AMR determinants, including Mykrobe[5] and TBProfiler[6], each with pros and cons. The software tool developed in our setting, tbtAMR (https://github.com/MDU-PHL/tbtamr), is a python package. It takes as input paired-end Illumina fastq files and implements TBProfiler using a custom database of mutations and generates an inferred antibiogram for clinical reporting (Figure 1).

Mutational catalogue

The mutational catalogue implemented within tbtAMR includes mutations from TB-Profiler[10] (v 4.0), Mykrobe[11] and the WHO catalogue[8]. The tbtAMR database consists of mutations and the antibiotics to which they confer resistance, as well as degree of inferred resistance (resistant or low-level resistant) and the confidence that the mutation is indicative of the degree of resistance for this prediction. Confidence scores are provided as High (odds ratio [OR] > 10), Moderate (1 < OR < 10) and Unconfirmed (0 < OR < 1), where OR is the OR data supplied by the WHO catalogue (Supplementary Table 1). Further details of database development and curation can be found in Supplementary methods.

Validation dataset

We included three data types in our validation dataset (Supplementary Figure 1). Firstly, we included sequences generated at MDU PHL and with phenotypic data generated at the Mycobacteria Reference Laboratory (MRL) (Supplementary Methods). Secondly, publicly available sequences were included, selected to be representative of all major global lineages and with phenotypic data available for first-line agents (with a subset also having data available for second-line agents). Lastly, we also used simulated genomic data to supplement the existing datasets. Paired-end sequence data was generated using TreeToReads software[12–15], directly from the H37rV strain (RefSeq accession NC_000962.3) or following introduction of 2–4 variants per 10,000 bases at known positions with an error-profile representative of the NextSeq500 instruments at MDU PHL. These simulated reads were mixed (Supplementary Figure 2) to simulate different allelic frequencies (0.01 to 0.99) across a range of average genome depth (10x to 200x).

Measuring the accuracy of Mtb genomic sequence recovery

118 For simulated genomes, we tested the recovery (identification) of introduced variants at a minimum
119 depth of 10X and 20X, meaning that to make a base call there was least 10 or 20 reads across each
120 position. For each position in the reference genome, a true positive (TP) result was observed if a variant
121 was recovered where one had been introduced, a true negative (TN) result was observed where the
122 same base as the reference was recovered where no variant had been introduced. A false positive (FP)
123 result was observed where a variant was recovered when no variant had been introduced, and a false
124 negative (FN) result was observed where a reference base was recovered but a variant was had been
125 introduced (Supplementary Figure 2).

126 We also used publicly available sequences to determine the concordance of variant detection in
127 sequences by tbtAMR with the approach described by Ezewudo *et al*. These sequences were used to
128 support the limit of detection (average genome depth) of the tbtAMR pipeline. To simulate different
129 quantities of DNA recovery, reads were randomly down-sampled to 0.8, 0.6, 0.4 and 0.2 of the original
130 population, in triplicate (using seqkit sample). The variants were considered concordant if those
131 identified from the down-sampled sequence were also identified in the original sample.

132 **Assessing suitability of MGIT sequences for inference of AMR**

133 To assess the utility of DNA generated from MGIT sequences for the interpretation of relationships and
134 the inference of AMR, we evaluated matched sequences generated from solid cultures (gold standard)
135 and MGITs for 66 primary samples (total of 161 sequences evaluated, as some samples had >1
136 sequence per culture type), where all sequences had passed the sequencing quality checks
137 (Supplementary Figure 1). To establish whether sequences generated from MGIT culture provide a
138 comparable degree of utility for identifying and interpreting relationships, we assessed the consistency
139 of variant detection and the pairwise distance between the matched sequences from MGIT culture
140 and solid culture.

141 **Analysis of inferred phenotypic DST and phylogenetic lineage from genomic data**

142 tbtAMR leverages the most recent definitions of phylogenetic lineage, including lineages 1–9 as well
143 as animal adapted species/sub-species[6]. Phylogenetic lineage was assessed by the concordance of
144 phylogenetic lineage determined by tbtAMR and compared with available public data[16]. The inference
145 of phenotypic DST from genomic sequence data was assessed by determining whether the presence
146 of a genomic resistance determinant, SNP (single nucleotide polymorphism) and/or
147 insertion/deletions correlated with the phenotypic DST results, with sensitivity, specificity, negative
148 predictive value (NPV), positive predictive value (PPV) and accuracy recorded (Supplementary Table 2).
149 A true positive (TP) was recorded when a mutation was present and the phenotype was resistant; a

150 true negative (TN) was recorded when no mutation was detected and the phenotype was susceptible;
151 a false positive (FP) was recorded when a mutation was present, but the phenotype was susceptible,
152 and a false negative (FN) was recorded when no mutation was detected and the phenotype was
153 resistant.

154 Results

155 **Validation of tbtAMR tool**

156 *Accuracy of sequence recovery by tbtAMR*

157 Firstly, to assess the impact of minimum depth for base calling, we compared FP SNPs and the
158 percentage of SNPs recovered across a range of average genome depth, maintaining the allelic
159 frequency at 100%. We observed a higher number of FP SNPs, mean 6.6 (95% confidence interval (CI)
160 3.9-9.3) when using the standard criteria of 10X, which may result in over identification of resistance
161 mechanisms. Increasing the minimum depth for base calling to 20X improved the number of FP SNPs
162 to mean of 1.1 (CI 0.7-1.5), with little impact on the percentage of introduced SNPs recovered
163 (Supplementary Figure 2). We further examined the impact of varying average genome depth and
164 allelic frequency on the ability of tbtAMR to accurately identify mutations in the simulated sequences.
165 Where average genome depth < 40 and allelic frequency < 10% sensitivity was only 2.1% (CI 1.48%-
166 2.66%) and False Discovery Rate (FDR) was 41.1% (CI 31.1%-51.2%). However, maintaining 20 reads
167 minimum depth for base-calling, an average genome depth of ≥40X and allele frequencies ≥10% gave
168 the best balance (Figure 2A and Supplementary Figure 4), with an overall variant identification
169 sensitivity of 96.3% (CI 95.5%-97.1%) and low FDR of 0.21% (CI 0.11%-0.31%). Hence, the minimum
170 acceptable read depth for running tbtAMR was set to ≥40X and minimum allele frequency of 10%.

171 tbtAMR performance was compared to results published for the UVP pipeline, identifying 285/296 of
172 the variants in the published dataset[17] (96.3% concordance). We determined that 3/11 discordances
173 were likely due to low frequency mutations identified by the tbtAMR pipeline, and the remainder likely
174 due to differences in SNP detection between tbtAMR and the UVP pipeline[17]. Down-sampling this
175 public sequence data demonstrated that at a median depth of ≥40X, ≥99% concordance between
176 SNPs detected in the original sequence by tbtAMR and the down sampled reads was observed,
177 indicating tbtAMR was able to reproducibly detect genomic AMR determinants in Mtb sequences
178 (Figure 2B).

179 *Sequencing from liquid culture (MGIT) compared to solid culture*

180 Firstly, we compared AMR determinants identified by tbtAMR of matched sequences from solid and
181 MGIT cultures. Using average genome depth of ≥40X and a minimum allele frequency of 10% as
182 established above, 93.9% (62/66) matched groups of sequences from MGIT and solid cultures
183 exhibited detection of the same genomic determinants of AMR. In two of the four discordant samples,
184 mutations were observed in *rrl*, a 23S rRNA gene (implicated in linezolid resistance). The remaining
185 two discordances were observed in groups where no single lineage could be detected in one sequence
186 from each group. It is possible that cases such as this reflect genuine in-host diversity (e.g. mixed
187 infections) and should not be excluded from analysis, however, it is also possible that observations
188 such as these are the result of laboratory error or contamination. In these cases, inferred AMR results
189 will be reported on an Interim basis, while awaiting investigation and resequencing (for detailed
190 discussion see below).

191 We routinely undertake identification of genomic relationships, for use in epidemiological
192 investigations, using a validated and accredited pipeline (https://github.com/MDU-PHL/bohra).
193 Therefore, it is important to establish the impact of using sequences from MGITs on this process. Thus,
194 we also examined the SNP distances between matched groups (pairs/trios of sequences from MGITs
195 and solid cultures) to determine the consistency of sequences between solid culture and MGITs from
196 the same primary samples. The median distance between sequences from the same primary sample
197 was 1 SNP (IQR +/- 3). Pairwise distances between matched sets fell within the 5 SNP threshold for
198 59/66 matched groups (89.4%); a further five matched groups fell within 12 SNPs (7.6%) and would
199 therefore be identified as genomically-related in our routine analyses (Figure 3). Two remaining groups
200 contained a single sequence each for which multiple phylogenetic lineages were observed, consistent
201 with mixed infection or laboratory contamination. For the purposes of interpretation of genomic
202 relationships, sequences where no single lineage can be identified will be failed and resequencing
203 undertaken.

204 *Accuracy of phylogenetic lineage calling by tbtAMR*

205 tbtAMR accurately identified lineages compared to lineages reported from public datasets[16], (99.2%
206 concordance). Of these discordant results (Supplementary Table 3), seven were cases where tbtAMR
207 identified two different phylogenetic lineages in the sequence. In each case, the reported lineage was
208 one of the lineages detected by tbtAMR, therefore these likely reflect mixed sequences, where tbtAMR
209 was able to identify the minor allele.

210 **Inference of phenotypic AMR from genomic data and discrepancy characterisation**

211 Many studies have explored the inference of phenotypic AMR from genomic data using various
212 tools[5,6,8,17,18]. We do not aim to recapitulate these high-quality studies here, but to assess the suitability
213 of tbtAMR in our setting for inference and reporting of AMR in Mtb for clinical and public health.

214 We began with a dataset of 3374 sequences from both publicly available datasets[16–18] and sequences
215 generated in-house (Supplementary Figure 1). Implementing the quality control thresholds and limits
216 of detection outlined above, we excluded 371 sequences, leaving 3006 sequences of sufficient quality
217 to include in the validation of tbtAMR for inference of phenotypic AMR (Figure 4A). This dataset
218 contained > 500 sequences for each of the common international lineages (Figure 4B).

219 The performance of tbtAMR in inferring phenotypic resistance to first-line drugs was excellent, with
220 accuracy > 95% across all first-line drugs (Table 2 and Figure 5A). For the purposes of validation for use
221 in a CPHL, investigation and resolution of such discrepancies needs to be undertaken. Discrepancies
222 observed in prediction of pyrazinamide, isoniazid and ethambutol have been observed in other studies
223 [REF] and are consistent with our observations.

224 Phenotypic tests based on both liquid and solid culture methods for moxifloxacin resistant have been
225 shown to have low levels of consistency and also inconsistent genomic mechanisms[19].Therefore for
226 validation of moxifloxacin, we only included samples with phenotypic data available generated in our
227 laboratories, to ensure that the comparisons made were consistent with methodology that was used
228 for reporting resistance in our setting. The tbtAMR pipeline was able to predict moxifloxacin resistance
229 phenotype from the detected genotype with very high accuracy (>98%), sensitivity (100%) and
230 specificity (>98%) (Table 2 and Figure 5B). The positive predictive value (85.7%) was limited by the low
231 proportion of resistance (genotypic and phenotypic) in the dataset (only 12 true positives and two
232 potential false positives).

233 tbtAMR predicted resistance to second-line and other drugs with performance comparable to other
234 studies[5,20]. The high number of false-negative results for prediction of phenotypic resistance (Table 2)
235 are likely to be due to incomplete understanding of the mechanisms of resistance to these drugs,
236 meaning that even when mutations are correctly identified, the functional impact is unknown and
237 therefore prediction of resistance is not possible.

238 **Report design**

239 Reporting of AMR from WGS data for Mtb can potentially directly impact patient management,
240 particularly when undertaken from MGIT culture with reduced turnaround times. Three key
241 parameters are reported for each drug to assist in clinical decision making: (i) presence or absence of

242 mutations including small indels and SNPs, (ii) confidence that this mutation is associated with drug
243 resistance, and (iii) the level of drug resistance conferred by this mutation.

244 We have leveraged the confidence levels from the WHO mutation catalogue and supplemented it with
245 an inferred phenotype interpretation of 'susceptible', 'low-level resistant' and 'resistant' to allow for
246 clinically informative reporting (Supplementary Table 1). These interpretation levels are based on
247 available laboratory evidence, supported by the literature for a particular phenotype.

248 As AMR genotypes and inferred antibiograms for Mtb were new to most clinicians, additional
249 interpretive comments were constructed for inclusion in reports – for example, explaining the
250 difference between confidence level and level of resistance in the inferred phenotype (Supplementary
251 Methods and Supplementary Files 1). Clinical and laboratory colleagues were consulted about the draft
252 report design to ensure the formatting and explanations were clear and unambiguous.

253 **Implementation process**

254 Once validation and report design were complete, Mtb genomic AMR inference using the tbtAMR tool
255 was implemented into existing genomic workflows, including standard operating procedure
256 documentation (SOP), staff training, incorporation into the laboratory information management
257 system, and testing of reporting and feedback loops (e.g. reporting of samples that failed QC).

258 External clients and stakeholders (diagnostic laboratories, clinicians, Department of Health/TB
259 program) were notified of the new test through existing channels, and the contact details for the
260 medical microbiologists were included on the reports to facilitate further discussions if required.

261 To address any discrepancies in phenotype and genotypic results, a multidisciplinary expert panel for
262 Mtb AMR was formed, meeting monthly to discuss individual cases and provide expert
263 recommendations to clinicians where discrepancies were identified. This provides the opportunity for
264 ongoing dialogue between the wet-lab microbiologists, clinicians, epidemiologists, medical
265 microbiologists and bioinformaticians to monitor for AMR determinants of interest, discuss individual
266 cases and discrepancies, and undertake prospective validation of novel or uncharacterised AMR
267 determinants.

268 In preparation for ISO-equivalent accreditation, revalidation and reverification processes were also
269 defined for tbtAMR (Supplementary Methods). Subsequently, the Mtb genomic AMR inference
270 workflow has now been accredited to ISO 15189:2022 standards.

Discussion

We have demonstrated here the development of a custom pipeline with a fit-for-purpose mutational catalogue, to maximise the balance between PPV and NPV for prediction of AMR in Mtb. Performance of inferred AMR using our process is consistent with or exceeds previously published results on similar datasets[5,16]. This pipeline has been robustly validated and implemented to ISO standards, with reverification strategies for ongoing improvement, and reporting processes put in place.

While there are many excellent tools available for detection of resistance mechanisms in Mtb, the outputs and/or databases and levels of interpretation were not strictly suited to reporting in a high-throughput CPHL. Reporting and data storage methods are amongst some of the considerations which prevent 'out-of-box' use of existing bioinformatics software. We assessed the two most popular existing software tools, TB-profiler and Mykrobe and elected to leverage aspects of TB-profiler to identify genomic variation, adding a custom collection of mutations. We then implemented reporting logic with interpretive comments specific for use in a clinical setting, based on our validation and supported by literature and laboratory evidence.

Clinical and public health laboratories operate rigorous quality systems to ensure that results are robust, accurate and fit-for-purpose. Comprehensive validation of new tests or pipelines is an essential component of this quality framework, optimising the performance metrics of the process prior to implementation. However, this can also be a dynamic process, as software and database updates occur regularly in the bioinformatics field. As such, it is important to establish a regular process for pipeline reverification before any updates or modification to the code base of tbtAMR or its dependencies are approved (Supplementary Figure 5). To maintain the integrity of tbtAMR, we utilise conda, a software management framework, implementing tbtAMR in a centralised conda environment, which is only updated upon successful testing of the proposed updates.

AMR determinants are often reported as a binary 'present/absent' variable, inferring a 'resistant' or 'susceptible' phenotype respectively, but this does not always accurately reflect the complexity of genotype-phenotype correlations. It is important to ensure that mutations used to infer resistance have solid evidence supporting a role as genomic determinants of resistance. The databases underlying both TB-profiler and Mykrobe have been extensively curated and incorporate many, although not all, of the variants described in the WHO catalogue. In addition to variants highly likely to confer resistance, our database also contains many mutations that are being investigated for their role in resistance. However, we have classified these mutations as 'Not reportable' since there is currently insufficient evidence for their causative role in resistance (Supplementary Methods). This allows us to provide meaningful reports to clinicians (based on high confidence mutations), whilst still being able

304 to prospectively monitor and validate additional mutations. This is particularly relevant for second-
305 and third-line drugs, such as para-aminosalicylic acid and cycloserine, as well as the newer critical
306 antimycobacterial drugs bedaquiline, delaminid and linezolid, where a lack of data, locally and globally
307 limits our ability to adequately validate inference of resistance to these drugs. Even for drugs which
308 are well studied, discrepancies can still occur, and further research and iterative validation will only
309 improve the performance of tbtAMR and other tools used by Mtb genomics community, ultimately
310 leading to improved patient outcomes.

311 tbtAMR represents a robust and accurate pipeline that uses high-quality open-source tools, a database
312 tailored to achieve the best balance between PPV and NPV for patient management, and simplified
313 outputs for clinical and public health microbiology. We have accredited this pipeline to ISO-standards
314 in our laboratory, and have provided the validation methods and dataset for use by other laboratories
315 endeavouring to make a similar transition. However, our results also highlight areas where further
316 research can enhance our knowledge of AMR determinants in Mtb, and the nuanced interactions
317 between determinants and phenotypes with new second-line Mtb drugs is a key priority. Further
318 enhancements of diagnostic processes, such as sequencing direct from samples, will allow us to
319 maximise the benefit of genomic technologies for patient management. Our ongoing engagement with
320 stakeholders, including researchers, clinicians and public health officials ensures that the processes
321 established can be improved and refined where needed to continue to improve patient management
322 and inform timely public health responses.

Figures and tables

326

327 **Table 1 – Performance of tbtAMR pipeline to predict phenotypic susceptibility for first- and second-line antimycobacterial drugs**

Drug	Accuracy (%, 95%CI)	Sensitivity (%, 95%CI)	Specificity (%, 95%CI)	PPV (%, 95%CI)	NPV (%, 95%CI)
Rifampicin (n= 2957)	99.29 (98.92, 99.54)	97.34 (95.17, 98.55)	99.57 (99.24, 99.76)	97.08 (94.85, 98.36)	99.61 (99.29, 99.79)
Isoniazid (n= 2926)	98.09 (97.52, 98.52)	97.86 (96.21, 98.8)	98.13 (97.51, 98.6)	91.79 (89.19, 93.81)	99.54 (99.17, 99.74)
Pyrazinamide (n= 2962)	96.35 (95.62, 96.97)	80.97 (76.23, 84.95)	98.15 (97.57, 98.6)	83.67 (79.06, 87.42)	97.78 (97.15, 98.28)
Ethambutol (n= 2965)	96.12 (95.36, 96.76)	95.08 (91.76, 97.1)	96.22 (95.44, 96.88)	71.1 (66.17, 75.59)	99.5 (99.15, 99.71)
Moxifloxacin (n= 170)	98.82 (95.81, 99.68)	100.0 (75.75, 100.0)	98.73 (95.5, 99.65)	85.71 (60.06, 95.99)	100.0 (97.6, 100.0)
Amikacin (n= 279)	96.77 (93.98, 98.29)	95.56 (89.12, 98.26)	97.35 (93.96, 98.86)	94.51 (87.78, 97.63)	97.87 (94.66, 99.17)
Kanamycin (n= 279)	96.77 (93.98, 98.29)	95.7 (89.46, 98.31)	97.31 (93.86, 98.85)	94.68 (88.15, 97.71)	97.84 (94.57, 99.16)
Streptomycin (n= 98)	89.8 (82.23, 94.36)	95.52 (87.64, 98.47)	77.42 (60.19, 88.6)	90.14 (81.02, 95.14)	88.89 (71.94, 96.15)
Cycloserine (n= 98)	81.63 (72.83, 88.05)	45.45 (29.84, 62.01)	100.0 (94.42, 100.0)	100.0 (79.61, 100.0)	78.31 (68.3, 85.82)
Capreomycin (n= 278)	97.12 (94.43, 98.53)	94.44 (87.65, 97.6)	98.4 (95.41, 99.46)	96.59 (90.45, 98.83)	97.37 (93.99, 98.87)
Ethionamide (n= 180)	89.44 (84.1, 93.14)	84.78 (71.78, 92.43)	91.04 (85.0, 94.8)	76.47 (63.24, 86.0)	94.57 (89.22, 97.35)
Para-aminosalicylic acid (n= 98)	95.92 (89.97, 98.4)	20.0 (3.62, 62.45)	100.0 (96.03, 100.0)	100.0 (20.65, 100.0)	95.88 (89.87, 98.38)

328

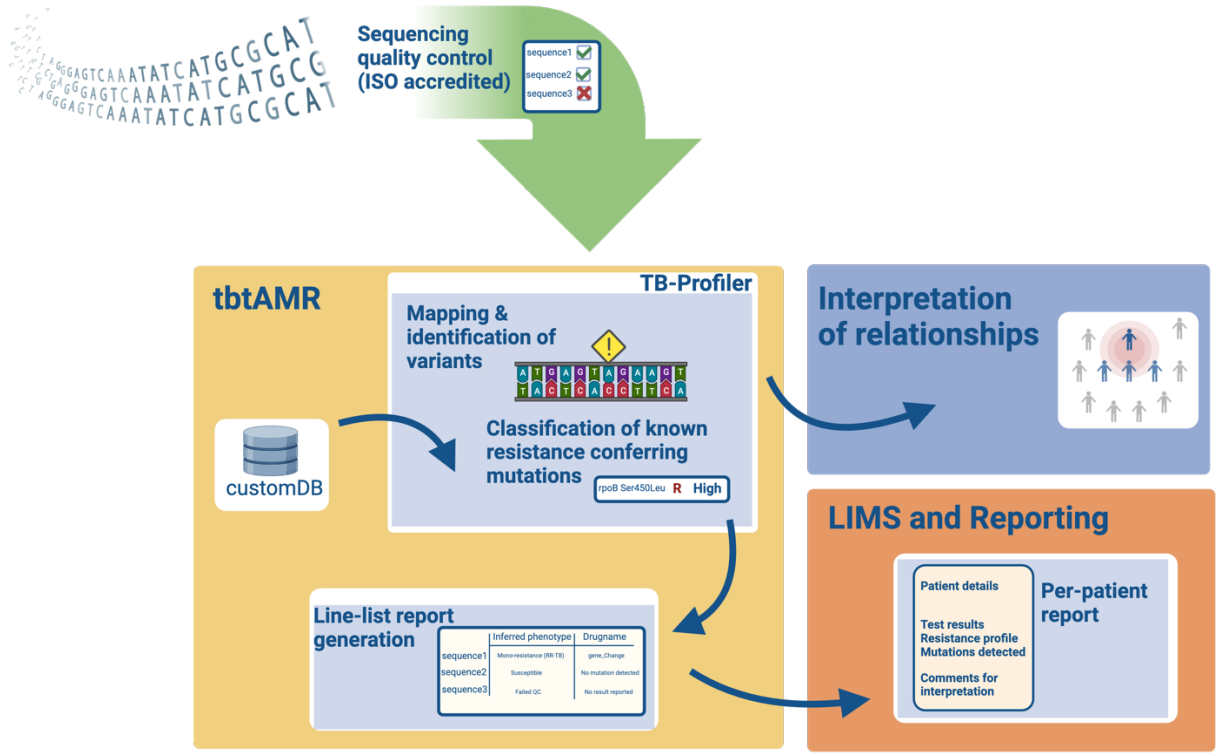


Figure 1 – Outline of data flow for the tbtAMR tool

tbtAMR takes paired-end fastq files that have passed sequencing quality control as input. TB-profiler is used to identify variants and known resistance-conferring mutations reported, based on the tbtAMR custom database. Using logic generated during data exploration, inferred phenotypes are recorded, and line lists are generated which can be bulk uploaded into the laboratory information management system (LIMS) for automated per-patient reports. In addition, the paired-end fastq files are also incorporated into the ISO accredited surveillance software utilised in our setting. ISO, International Standards Organization; DB, database.

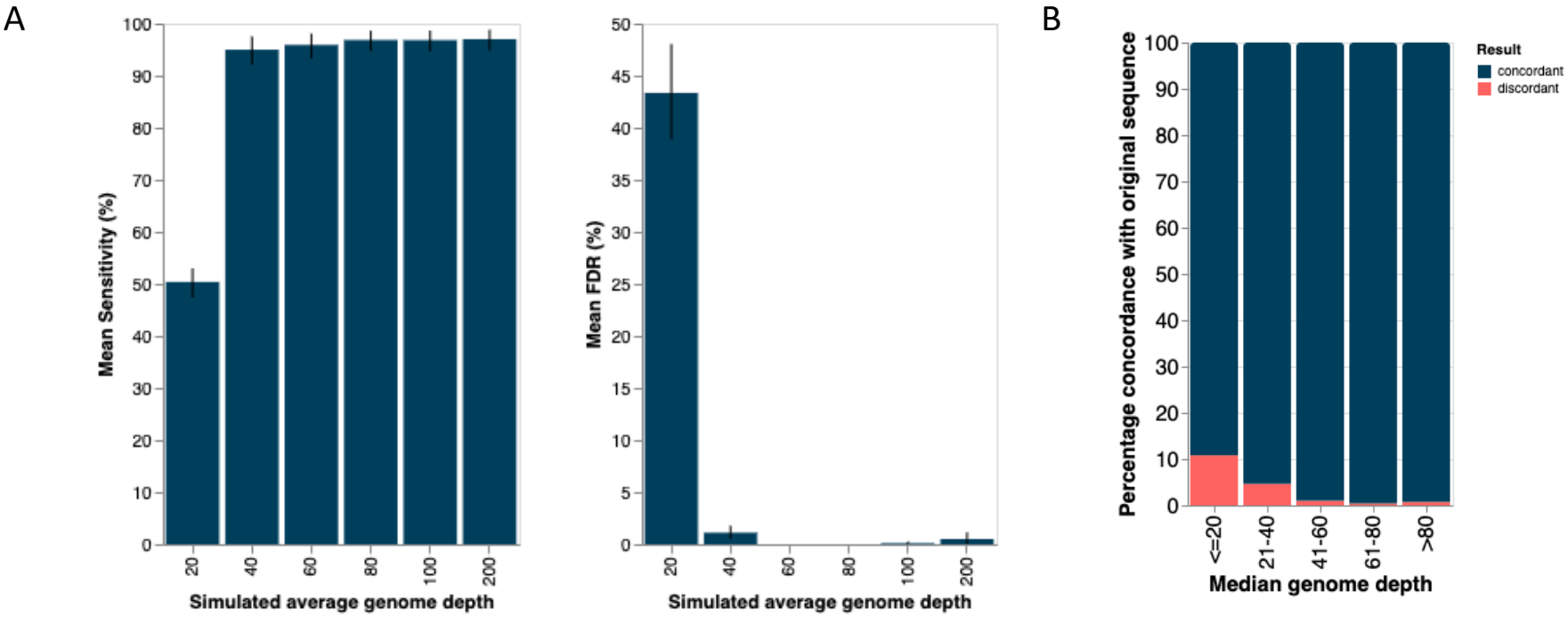


Figure 2 – Performance of tbtAMR for identification SNPs in *M. tuberculosis* genomes

A) Simulated paired-end fastq (read) files were used to assess the performance of tbtAMR in recovering introduced variants. Using a minimum read criteria of 20X for individual base calling, tbtAMR had SNP calling sensitivity (left panel) of ≥95% and false discovery rate (FDR) (right panel) of ≤1.0% when simulated average genome depth was ≥40X and allelic frequency ≥10%. B). Recovery of resistance-conferring mutations at varying simulated average genome coverage levels was compared to the resistance-conferring mutations identified in the original sequence (red indicates discordance with original sequence, blue indicates concordance with original sequence).

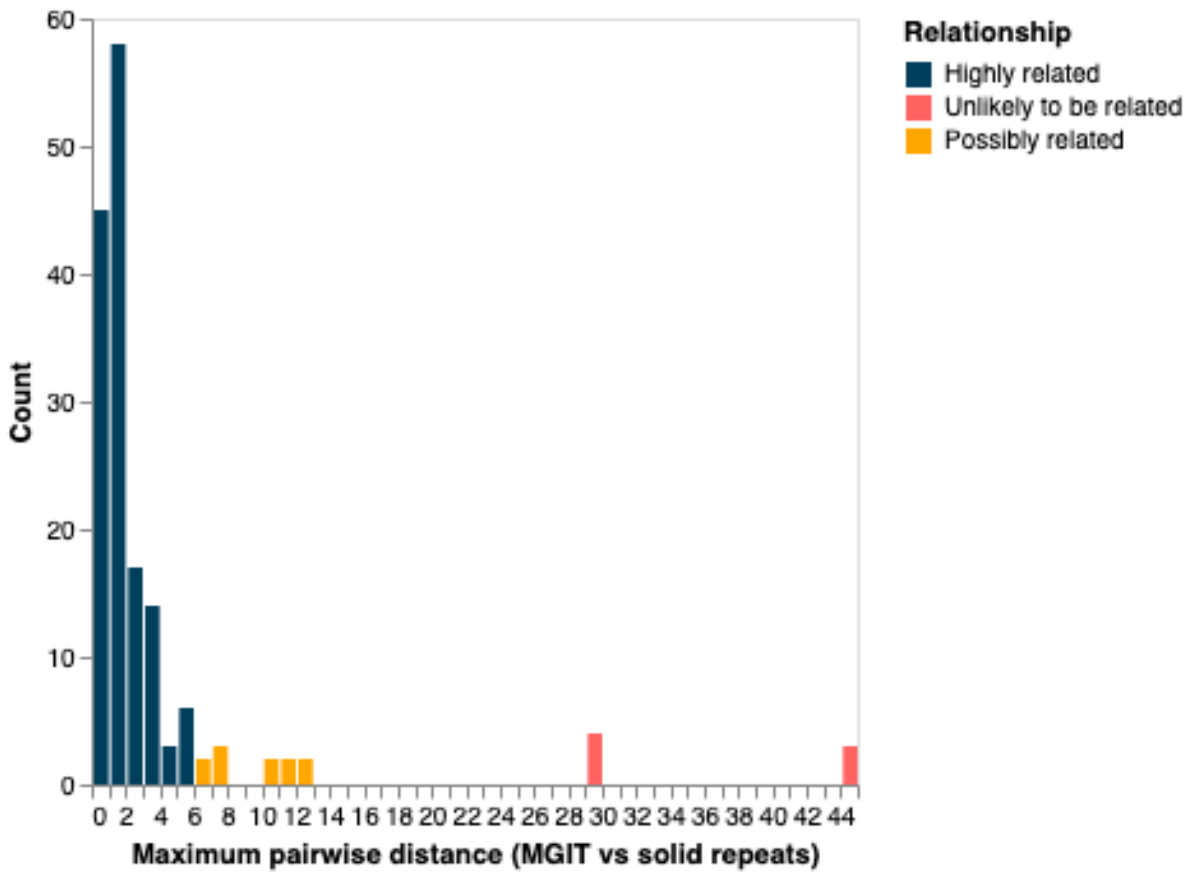


Figure 3 – Distance between sequences from matched MGIT and solid culture.

Pairwise SNP distance was calculated between sequences obtained Mycobacterial Growth Indicator Tubes (MGITs) and solid culture of the same clinical sample and the distribution plotted. Blue indicates sequences which would correctly be called highly genomically related, orange indicates sequences which would be considered possibly related and red indicates matched pairs where the pairwise distance would indicate no recent genomic relationship (evidence of mixed sequences; see Results).

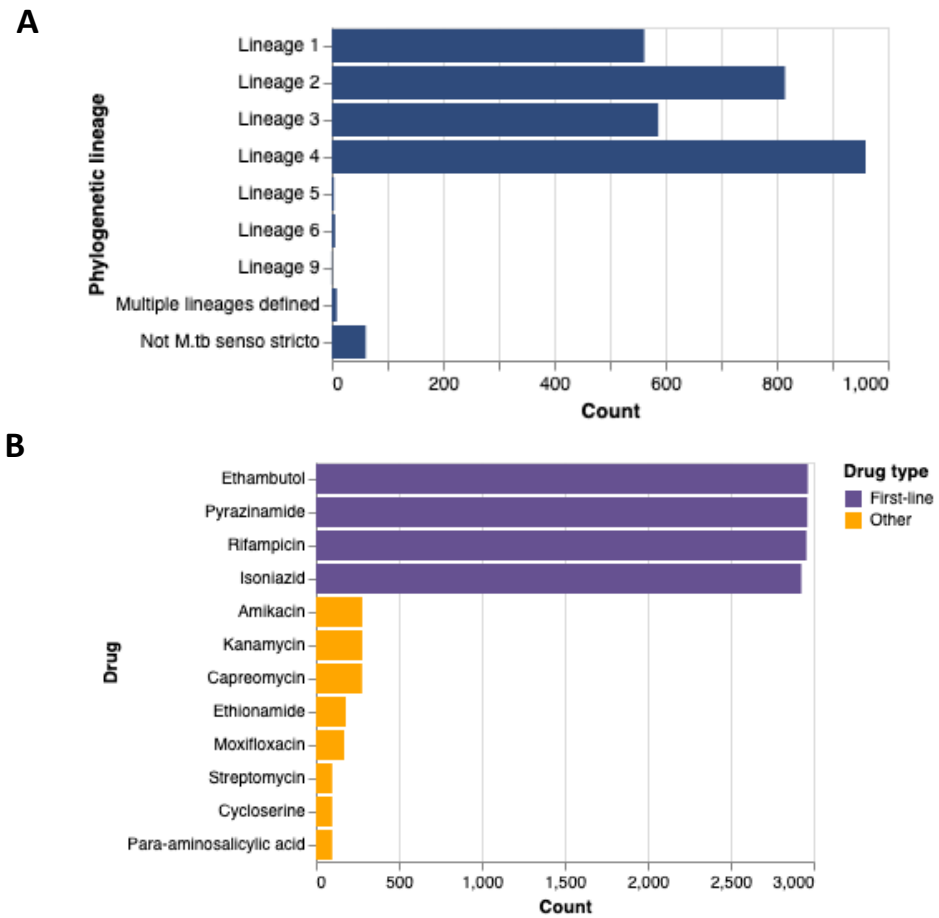


Figure 4 – Lineages and phenotypic antimicrobial resistance profiles represented in validation dataset

3016 Mtb complex sequences were used to validate the inference of AMR. A) Phylogenetic lineage was determined using tbtAMR pipeline, and grouped by lineage. Non-Mtb *sensu stricto* lineages were grouped together. B) The validation dataset represented by the phenotypic resistance for each drug.

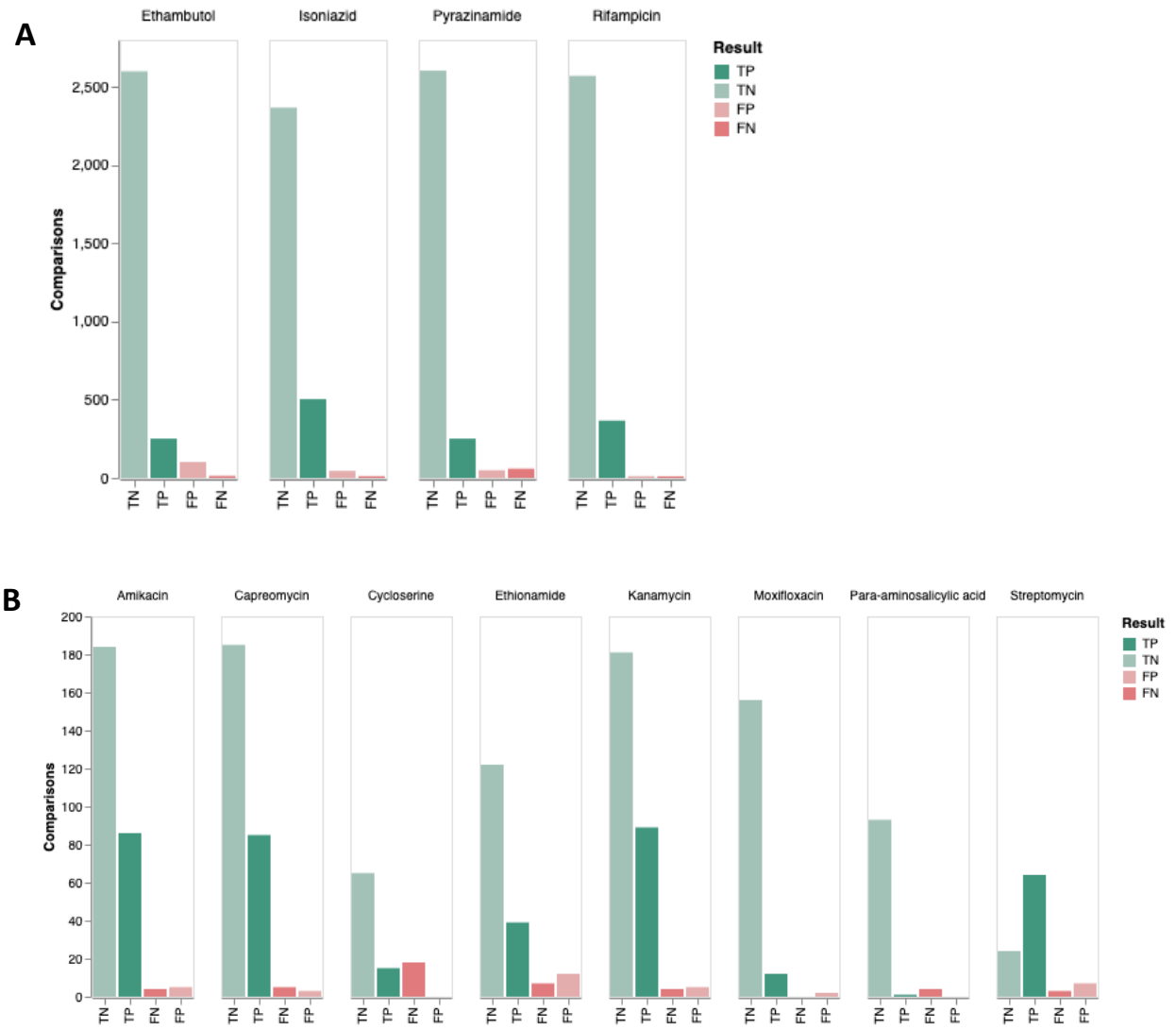


Figure 5 – Performance of tbtAMR inference of phenotypic DST

Inferred phenotypes predicted by the tbtAMR pipeline was compared to phenotypic susceptibility data for and results of the comparison classified into TP, TN, FP and FN for A) first-line drugs and B) other drugs.

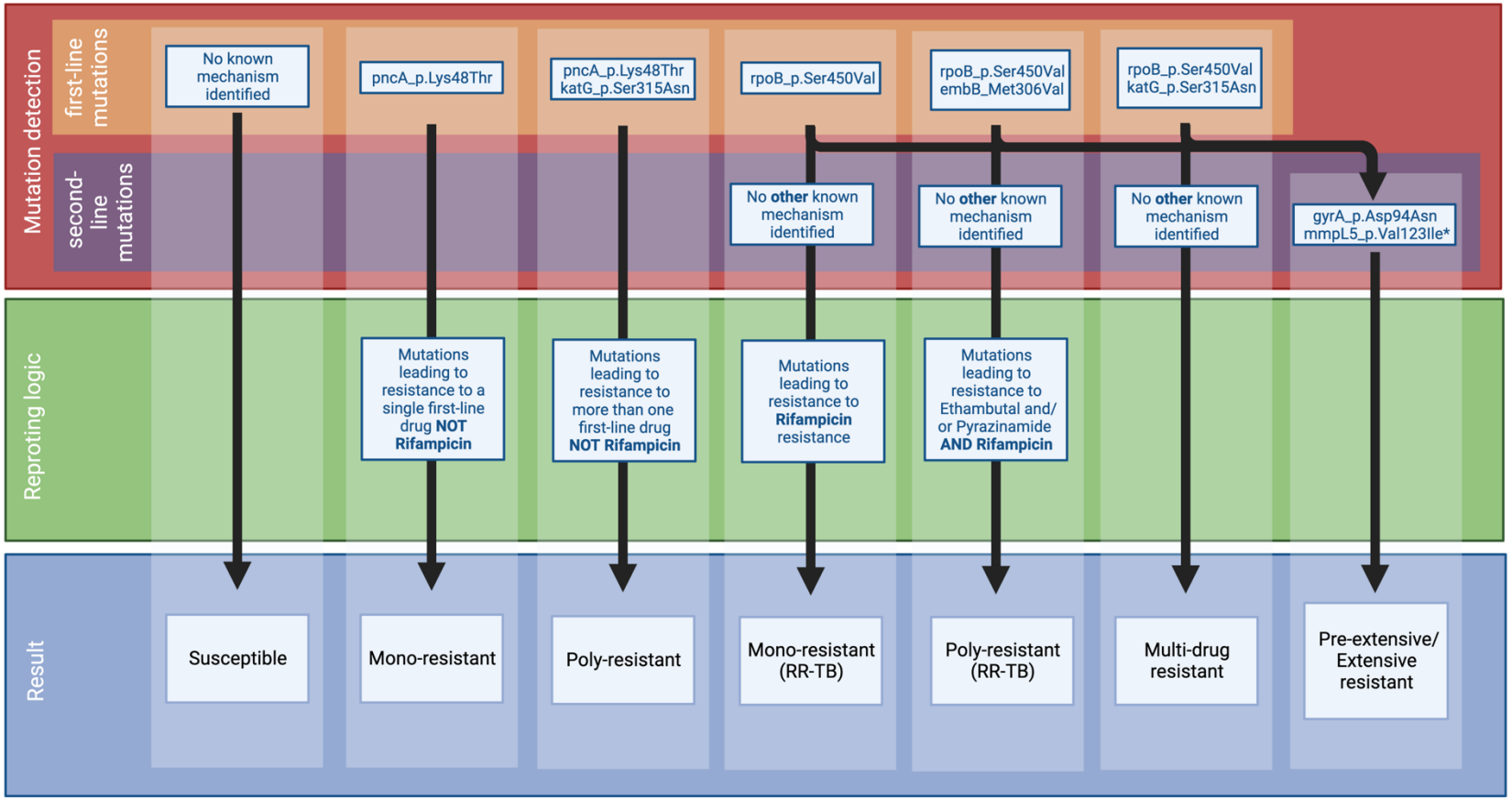


Figure 6 – Reporting of predicted drug resistance profile

Drug resistant profile is based on the WHO guidelines[21]. Note that Pre-extensive (Pre-XDR) and Extensive Drug Resistance (XDR) cannot yet be differentiated from genomic data (distinguished by bedaquiline and linezolid resistance) and are hence reported as a single group.

Ethical approval

Ethical approval was received from the University of Melbourne Human Research Ethics Committee (study number 1954615).

Acknowledgements

We thank all the laboratory staff at the Mycobacterial Reference Laboratory, Victorian Infectious Disease Reference Laboratory (VIDRL) and the Microbiological Diagnostic Unit Public Health Laboratory (MDU PHL) who undertook receipt, diagnostics, culture, DNA extraction and sequencing of the material used in this publication and the diagnostic laboratories who diligently provide samples for testing, and the Victorian TB Program and Department of Health Victoria who fund these services. We also thank the developers and maintainers of the open-source software used and cited in this publication.

383

384 # Supplementary Data

385 ## Methods

386 *Bacterial culture*

387 Clinical samples collected for suspected TB are processed at diagnostic laboratories throughout the
388 state of Victoria, Australia (population 6.71 million in 2022), where routine culture is performed using
389 both broth (mycobacterial growth indicator tubes (MGIT)) and solid culture media. In Victoria, primary
390 samples are cultured both in-house and within external laboratories. Samples with acid-fast bacilli
391 detected are referred to the Mycobacterial Reference Laboratory (MRL) at the Victorian Infectious
392 Diseases Reference Laboratory (VIDRL) for identification and phenotypic susceptibility testing. Broth
393 cultures (MGIT) are sub-cultured onto solid media to provide sufficient material for downstream
394 processes.

395 *DNA extraction from MGITs and solid cultures*

396 DNA was extracted from solid cultures and broth cultures (MGIT) as previously described[22] with minor
397 modifications. In brief, 3 x 1µl loops of culture were resuspended in 700µL TE and heat killed at 95℃
398 for 15minutes. For MGITs, 1ml aliquots were heat killed followed by centrifugation and resuspension
399 of the pellet into 700 µL TE. Cells were lysed through mechanical disruption and DNA precipitated with
400 ethanol and sodium acetate followed by elution into EB buffer (QIAGEN).

401 *Whole genome sequencing (WGS)*

402 Extracted DNA from solid or broth (MGIT) cultures was transferred to the Microbiological Diagnostic
403 Unit Public Health Laboratory (MDU PHL) for WGS, starting with NexteraXT (Illumina) library
404 preparation according to manufacturer's instructions, then paired-end short-read sequencing on
405 Illumina NextSeq500/550 platforms.

406 *Phenotypic DST*

407 Phenotypic drug susceptibility testing was set up for first line drugs in the BACTEC MGIT 960 system
408 according to WHO guidelines[23]. If resistance at the critical concentrations (rifampicin 0.5µg/mL,
409 isoniazid 0.1µg/mL, ethambutol 5.0µg/mL and pyrazinamide 100µg/mL) was detected, test was
410 repeated and simultaneously second line drugs were set up (amikacin 1.0µg/mL, capreomycin
411 2.5µg/mL, ethionamide 5.0µg/mL, kanamycin 2.5µg/mL, ofloxacin 2.0µg/mL, moxifloxacin
412 0.25/1.0µg/mL and isoniazid 0.4µg/mL).

413

414 *Reverification strategy*

415 It is important to maintain the integrity of any process where the outcome is to be used in informing
416 public health reporting and patient management. Many bioinformatics tools and databases are
417 updated frequently and whilst it may be desirous to always have the most up to data versions, it is also
418 important to ensure that no degradation of quality results occurs as a result. Therefore, it is important
419 to have a robust reverification strategy in place to assess the impact of any changes or updates. Any
420 updates to the underlying tools of tbtAMR, including TB-Profiler and its dependencies or new
421 mutations, the potential impact will first be assessed, using the most appropriate dataset
422 (Supplementary Figure 5).

423 1. If updates include changes to the methodology used to detect underlying detection of
424 variants, the performance of tbtAMR to identify accurate sequence will be assessed using the
425 simulated dataset described above.

426 2. If updates involve changes to the database, the performance of tbtAMR to predict AMR will
427 be assessed using sequences from samples submitted to the Victorian MRL.

428 3. If any modifications impact the way TB-profiler is implemented within the tbtAMR pipeline, or
429 the reporting logic of tbtAMR the sequences from sample submitted to Victorian MRL and
430 public datasets where phenotypic and sequence data are available will be used to confirm that
431 the results are consistent with the original validation results.

432 Any degradation of any performance metric compared to the original validation will be assessed to
433 determine the impact on clinical reporting. Some discordances may result in improvements to
434 prediction of AMR (increases in sensitivity and/or specificity etc). Whilst others may result in changes
435 to interpretation, such as level of resistance or confidence in the prediction. In rare cases, updates
436 could lead to a reduction in the confidence in results, such as failure to detect variants and/or incorrect
437 genomic DST result, in which case the updates will be rejected, and the existing versions retained.

438 *Cascade reporting from LIMS*

439 Phylogenetic lineage and predicted drug resistance profiles are reported, consistent with the WHO
440 classifications[21]. Pre-extensive drug resistance (pre-XDR) and XDR are reported together, as resistance
441 to bedaquiline and linezolid cannot yet be easily inferred from genomic data. Rules for reporting can
442 be defined by the user; in our lab, antimycobacterial drugs are reported in a cascade fashion. Second-

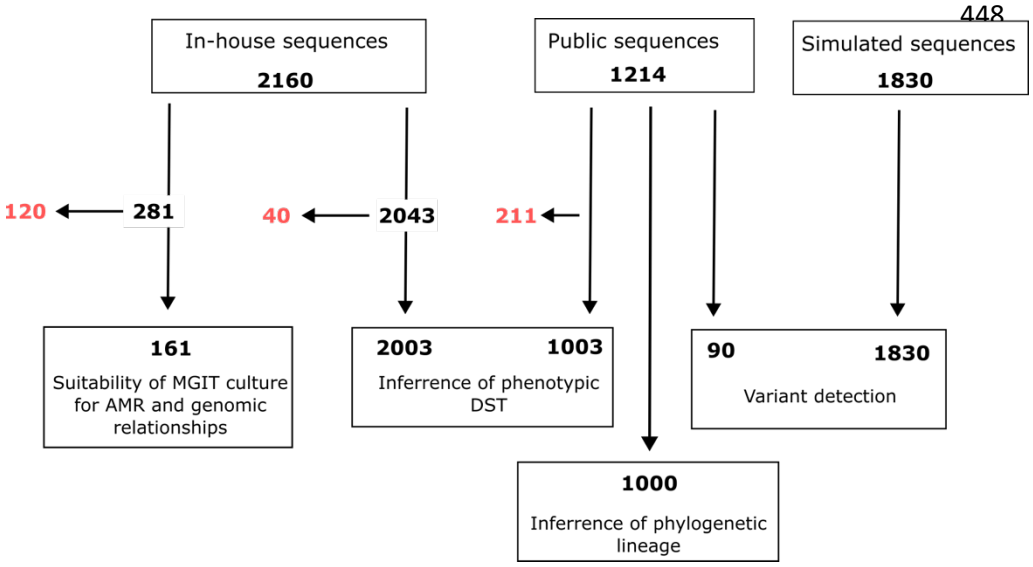
443 and third-line agents (where validated) are reported if MDR-TB, RR-TB or Pre-XDR/XDR-TB profiles are
444 identified.

445

446

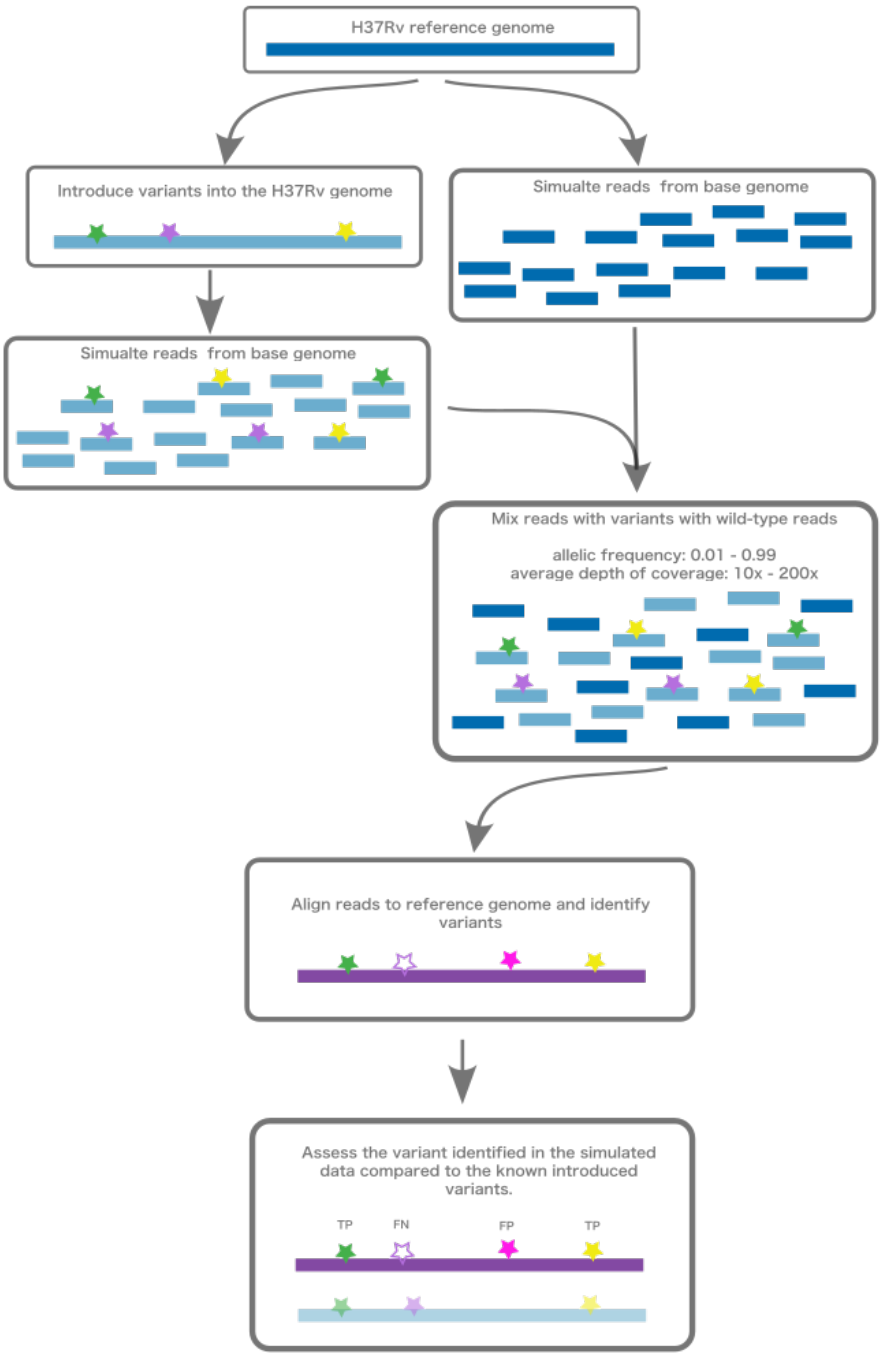
447 **Figures and tables**

448



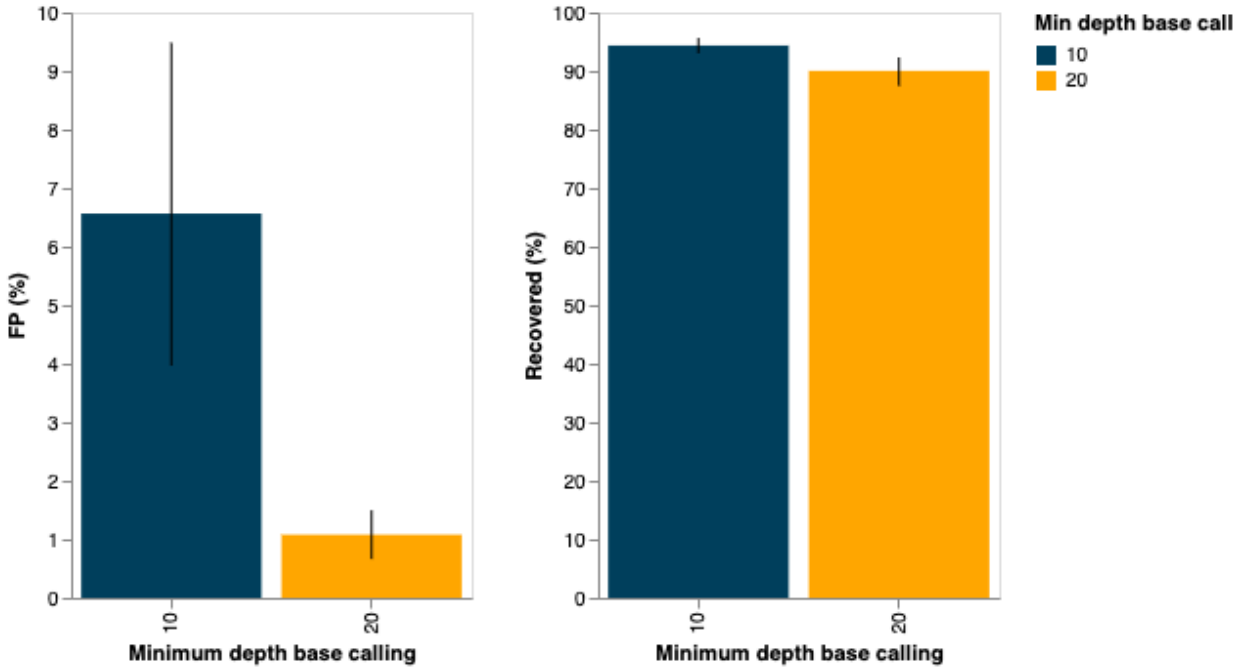
Supplementary Figure 1 – Validation dataset

Data generated in-house at MDU, downloaded from public datasets and also simulated data were used to validate variant detection, appropriateness of sequences derived from MGIT and genomic DST. Red indicates the number of sequences excluded from inclusion due to the failure to meet quality requirements.



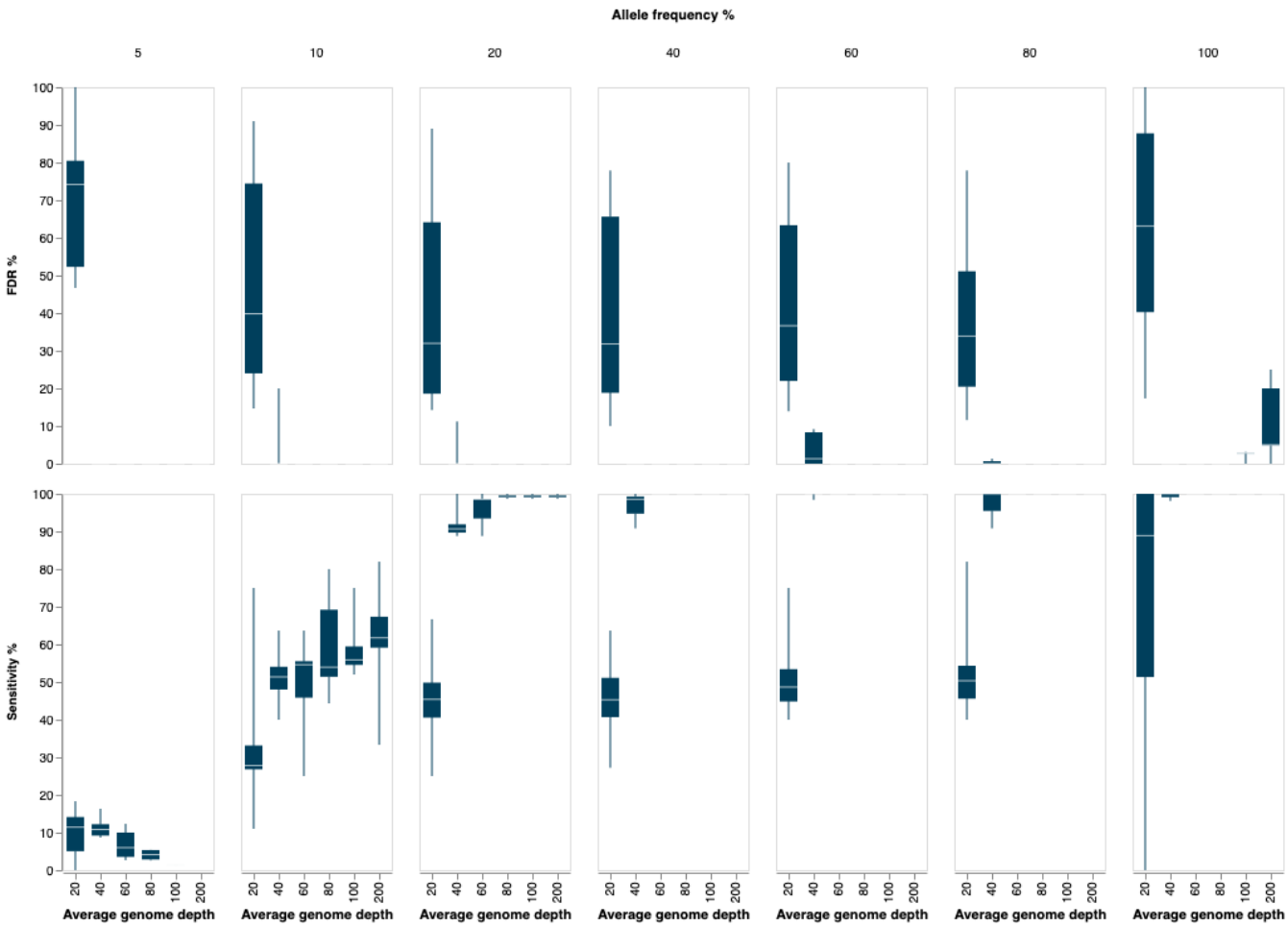
Supplementary Figure 2 – Generation and analysis of simulated reads

Simulated reads were generated to mimic the potential mixed alleles observed in Mtb sequencing. Variants were introduced (or not) into the H37rv reference at known positions and then mixed together at different proportions across a range of coverages. The simulated sequences were then analysed using the tbtamr pipeline and the recovery of the introduced variant was assessed. A TP result was observed where a introduced variant was recovered, a FP result was observed where a variant was reported that was not known to be present, and a FN result was observed where an introduced variant was not recovered



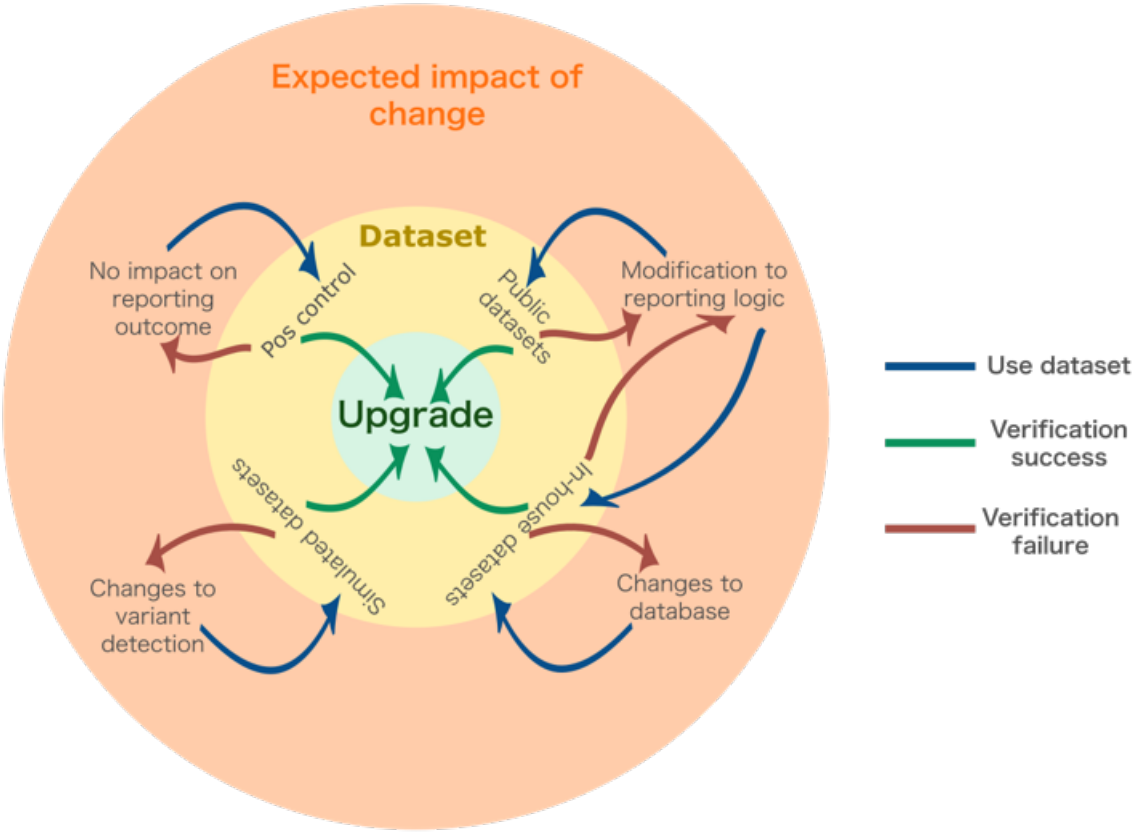
Supplementary Figure 3 – Impact of minimum read depth on performance of tbtAMR to recover SNPs from Mtb sequences

To assess the impact of minimum read depth for base calling, the percentage of A. FP and B. Introduced SNPs recovered by tbtAMR were calculated across a range of average genome depths from 10X to 200X at an allelic frequency of 100%.



Supplementary Figure 4 – Impact of genome depth and allelic frequency on performance of tbtAMR to recover SNPs from Mtb sequences

The FDR % (upper panel) and Sensitivity % (lower panel) of SNP recovery was assessed by varying the allelic frequency from 5 to 100 % and the average genome depth from 20 – 200X, whilst maintaining a minimum read depth of 20X for base calling.



Supplementary Figure 5 – Reverification process

Potential impact of proposed changes are shown in the outer circle and can include changes to the database, variant detection, modifications to reporting logic. Different datasets, shown in the middle circle, are required to address the different impact. If the result of the verification is deemed a success, then the update may proceed, however if the verification fails, then the update will not proceed and cause of failure established.

469
470 **Supplementary Table 1 – Generation of confidences and resistance levels for tbtAMR database**

Odds ratio	Evidence for low-level resistance	Reported parameters	
		Confidence	Resistance level
OR≥10	NA	High	Resistant
1≤ OR <10	Yes	High	Low-level resistant
1≤ OR <10	No	Moderate	Resistant
OR<1	NA	Unconfirmed	Resistant

471
472
473

Supplementary Table 2 – Definitions of quality metrics used

Metric	Definition
FDR	$\frac{False\ Positive}{True\ Positive + False\ Positive}$
PPV %	$\frac{True\ Positive}{True\ Positive + False\ Positive}\ x\ 100$
NPV %	$\frac{True\ Negative}{True\ Negative + False\ Negative}\ x\ 100$
Sensitivity %	$\frac{True\ Positive}{True\ Positive + False\ Negative}\ x\ 100$
Specificity %	$\frac{True\ Negative}{True\ Negative + False\ Positive}\ x\ 100$
Accuracy %	$\frac{True\ Positive + True\ Negative}{True\ Positive +\ True\ Negative + False\ Negative + False\ Positive}\ x\ 100$

477 **Supplementary Table 3 – Discordant Phylogentic lineage**

478 Phylogenetic lineages reported by tbtAMR were compared to those published, with only 1 of the 8
479 discordant results not being due the detection of mixed lineages by tbtAMR.

Accession	tbtAMR lineage	Published lineage[16]
ERR2513557	lineage1;lineage4	lineage1
ERR067636	lineage2;lineage4	lineage4
SRR6339653	lineage4	lineage1
ERR2510523	lineage3;lineage4	lineage3
ERR067732	lineage2;lineage4	lineage4
ERR2515255	lineage2;lineage4	lineage2
ERR2512421	lineage3;lineage4	lineage3

480

481

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