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 (WGS) is increasingly contributing to the clinical management of tuberculosis. Whilst the availability of bioinformatic tools for analysis and clinical reporting of *Mycobacterium tuberculosis* sequence data is improving, However, there remains a need for accessible, flexible bioinformatic tools that can be easily tailored for clinical reporting needs in different settings and are suitable for accreditation to international standards.

Methods We developed tbtAMR, a flexible yet comprehensive tool for analysis of *Mycobacterium tuberculosis* genomic data, including inference of phenotypic susceptibility and lineage calling. Validation was undertaken using local and publicly-available 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.

Findings tbtAMR accurately predicted lineages and phenotypic susceptibility for first- and second-line drugs, with equivalent computational and predictive performance compared to other bioinformatics tools currently available. tbtAMR is flexible with modifiable criteria to tailor results to users' needs.

Interpretation The tbtAMR tool is suitable for use in clinical and public health microbiology laboratory settings, and can be tailored to specific local needs by non-programmers. We have accredited this tool to ISO standards in our laboratory, and it has been implemented for routine reporting of AMR from genomic sequence data in a clinically relevant timeframe. Reporting templates, validation methods and datasets are provided to offer a pathway for laboratories to adopt and seek their own accreditation for this critical test, to improve the management of tuberculosis globally.

Funding Victorian Government Department of Health; Australian National Health and Medical Research Council and Medical Research Futures Fund.

Research in context

Evidence before this study

We searched PubMed for studies using the search terms: “Mycobacterium tuberculosis”, “clinical”, “bioinformatics”, “genomics”, “drug resistance (OR antimicrobial)”, published prior to 31st July 2024 without language restrictions (n=258). We considered all studies from this search that used genomics to infer likely drug-resistance in *M. tuberculosis*. Many of these studies highlight the challenges in making meaningful interpretations from genomic data for the purpose of inferring AMR for clinical applications. Despite the development of bioinformatics tools and compilation of catalogues of resistance conferring mutations, few of these studies directly address the challenges specific to implementation of a whole genome sequencing (WGS) and bioinformatics pipelines to deliver validated and accredited results for use in clinical applications and none provide sustainable solutions.

Added value of this study

To the best of our knowledge, this study is the first to detail practical solutions to challenges to validating and accrediting the routine inference of AMR from WGS data for clinical applications for treatment of *M. tuberculosis*. We developed, validated and accredited a bioinformatics tool, tbtAMR, which is robust and flexible, using a data-driven approach to inferring resistance, allowing for simple customisation of behaviour on a large collection of in-house data supplemented with publicly available data. Furthermore, we have made this pipeline and the accompanying data available for use by the wider public health community to aid others in implementing similar programs. Consultation with infectious disease clinicians, microbiologists and epidemiologists has allowed us to develop a robust workflow, that encompasses sequencing, analysis, interpretation, reporting, discrepancy resolution and ongoing verification.

Implications of all the available evidence

This study addressed the specific needs of reporting genomic AMR for *M. tuberculosis* in a clinical and public health laboratory. Furthermore, this work makes available a pipeline and data for laboratories to utilise to implement genomic AMR for *M. tuberculosis*. Through the use and further development of publicly available and open-source bioinformatics software,

90 we have demonstrated the feasibility of clinical reporting of genomic AMR results for *M.*
91 *tuberculosis* in a low-incidence high-income setting. We believe that this work lays the
92 foundation for making reporting of genomic AMR for *M. tuberculosis* more accessible to public
93 health and reference laboratories in general.

Introduction

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

Treatment of TB disease is lengthy and expensive, requiring combination therapy with at least two antimycobacterial drugs prescribed for a minimum of six months.[2,3] This is usually commenced with four antimycobacterial drugs ('first-line agents'): rifampicin, isoniazid, pyrazinamide and ethambutol, followed by rationalisation to two agents (rifampicin and isoniazid, if susceptible). If drug resistance is detected, an alternative regimen including second- and third-line agents will be used (Supplementary Figure 1 and Table 1).

The slow growth of Mtb *in vitro* means that clinicians treating patients with TB must choose treatment regimens before phenotypic drug susceptibility test (DST) results are available, which can take up to two months[4] in many settings. Whole genome sequencing (WGS) has the advantage of providing comprehensive results from an Mtb isolate in a single test (rather than individual tests for species identification, lineage, and drug susceptibility), potentially significantly reducing turnaround time to DST for both first-line and other antimycobacterial drugs, especially when undertaken from Mycobacteria Growth Indicator Tube (MGIT) culture. In addition, WGS can provide increased reliability of resistance detection for some drugs, due to known challenges with phenotypic reproducibility, such as ethambutol and pyrazinamide[5]. WGS data is also being used to provide support for outbreak investigation and contact tracing[6] (Figure 1A). Therefore, the use of routine WGS for Mtb has the potential to improve patient management in a clinical setting and public health outcomes, and new Australian national guidelines recommend routine use and reporting of findings for these reasons[7].

Despite the urgent need for routine identification of Mtb AMR from genomic data to inform therapy and the wealth of available bioinformatic resources, few clinical and public health laboratories (CPHLs) globally have processes in place to routinely report AMR from WGS data for Mtb. Many factors need to be considered when implementing such an AMR detection program, particularly where patient management may be impacted, including performance, ease-of-use and flexibility of tools in predicting drug-resistance, the impact of laboratory methodologies and existing processes, such as existing bioinformatics pipelines, reporting structure, CPHL data management, and test accreditation by national accreditation bodies to

125 International Organization for Standardization (ISO) standards (ISO15189:2022).[8] Laboratories
126 seek accreditation to ISO standards to demonstrate that they are able to produce robust,
127 reproducible and high-quality laboratory services; the process of obtaining accreditation, and
128 the difficulties in accrediting genomic tools, is covered in depth elsewhere[9].
129 There are many high-quality and established bioinformatics tools available for identification
130 of genomic determinants of AMR in Mtb, including Mykrobe,[10] TB-Profiler[11] and ariba[12], with
131 others being recently evaluated[13]. Additionally, a second version of the WHO Mtb Mutation
132 Catalogue[10,14] has now been released with an expanded catalogue of mutations for second-
133 and third-line drugs (Groups A-C Table 1), as well as guidance for interpreting complex
134 genomic interactions. Whilst this additional information is welcome and informative for
135 detailed analysis, clinical reporting often requires a more pragmatic approach as clinicians may
136 find this commentary difficult to interpret, preferring more binary results
137 (Resistant/Susceptible) where possible. For reasons such as this, users may wish to modify the
138 protocols, or 'rules', for interpretation of each variant. Alternatively, they may wish to add
139 other mutations of local interest into the resistance database used for analysis, or change the
140 way that mutations are classified (for example, swapping 'Susceptible' for 'Wildtype')
141 according to their local needs. Most of the currently used tools for Mtb genomic DST (gDST)
142 do not currently allow users to make similar changes without a bioinformatician to modify
143 existing code, limiting the ability of clinical and public health labs, as well as researchers, to
144 adapt tools to meet local needs. Furthermore, in high-throughput sequencing labs, WGS may
145 be undertaken in large batches with existing workflows and pipelines in place, therefore
146 bioinformatics tools need to be able to be easily incorporated into these existing pipelines, be
147 straightforward to maintain and for any updates to be assessed easily by reverification.
148 Here we describe the validation of a real-time routine analysis tool for inference of phenotypic
149 resistance in Mtb, based on detection of genomic variation, in a clinical and public health
150 microbiology setting. We have subsequently gained accreditation to ISO standard 15189:2022
151 (Clinical Laboratories) for this tool, tbtAMR, for clinical and public-health reporting of genomic
152 AMR detection in Mtb. The pipeline and validation approach are made available here for other
153 laboratories globally to aid in the transition to WGS for genomic AMR reporting.
154

155 # Methods

156 **tbtAMR design**

157 Many software options exist for detection of AMR determinants in Mtb sequences, including
158 Mykrobe[10] and TBProfiler[11], each with pros and cons. When developing a stand-alone tool,
159 we considered that the process must be easy to incorporate into existing laboratory workflows
160 (Figure 1A), have flexibility in key outputs, and to be easily maintained to expedite any
161 updates, ideally by non-developers. These were noted limitations of existing tools for use in a
162 CPHL, where software maintenance and updates can be challenging. In addition, reporting of
163 AMR from WGS data for Mtb will directly impact patient management. Thus, the results
164 generated should be suitable for reporting in a clinical setting with a layer of interpretation
165 that is fit-for-purpose, not reporting raw (unfiltered) results unless required for a specific
166 purpose. In tbtAMR, we report four key parameters to assist in clinical decision making: (i)
167 presence or absence of mutations including small insertions or deletions (indels), large
168 structural changes, gene loss (or loss of function) and SNPs, (ii) confidence that this mutation
169 is associated with drug resistance, (iii) the level of drug resistance conferred by this mutation
170 (high or low), and (iv) the overall resistance profile for the isolate based on the resistance
171 inferred to individual drugs (e.g. based on WHO definitions).

172 tbtAMR (https://github.com/MDU-PHL/tbtamr), is a pure python package and can be used as
173 a stand-alone tool with minimal dependencies, taking an annotated variant file (vcf) to
174 generate an inferred antibiogram and resistance profile based on user-defined criteria (Figure
175 1B). The recently updated WHO mutation catalogue for Mtb (version 2) is currently
176 implemented as the default mutational catalogue within tbtAMR[14] with its accompanying
177 guidelines documentation[15] informing criteria for classification. However, a user-defined
178 catalogue of mutations can also be supplied, along with bespoke criteria for interpretation
179 and classification according to a user's needs, allowing for increased flexibility and ease of
180 update.

181 Providing reportable interpretations of the likely resistance to each drug is a key requirement
182 of tbtAMR. Unlike many approaches, tbtAMR does not rely on hard coded or installed library
183 files to undertake this interpretation, but allows the user to choose between a default library
184 and user-modified files. tbtAMR relies on four accessory files (detailed below and Figure 1B,
185 and available at[16]): (i) a mutational catalogue or database of resistance mechanisms; (ii) rules

186 for interpreting resistance (such as categorisation as susceptible/resistant for each drug); (iii)
187 rules for reporting classification of resistance profile (such as 'fully susceptible' or 'MDR-TB');
188 and (iv) a config file defining the structure of the mutational catalogue and report outputs.

189 - *Mutational catalogue* – a CSV file containing the drugs, genes, variants and confidence
190 levels for calling drug resistance. The supplied default file is based on the WHO
191 catalogue version 2[15].

192 - *interpretation_criteria.csv* – this CSV file includes rules to interpret information
193 from the mutational catalogue when a variant is detected. The supplied default
194 interpretation rules file is based on the additional commentary supplied with
195 the WHO mutational version 2 catalogue (see Supplementary File 1). There are
196 two types of rules: 'default rules' applied broadly to most mutations, and
197 'override rules' for exceptions and edge cases. 'Default rules' describe rules for
198 interpretation of reportable drug resistance in most circumstances; for
199 example, for a certain drug, if a variant associated with resistance (or
200 associated with resistance – interim) is present, then report 'Resistant' for the
201 corresponding drug. Only drugs which have a default rule will be reported by
202 tbtAMR, allowing the user to control which drugs are reported. 'Override rules'
203 provide additional rules to describe interpretation of edge cases or exceptions
204 to the default rules, hence overriding the default rule. For example, when a
205 specific variant is detected, report "Low-level resistant" based on commentary
206 in the WHO Catalogue. Users may supply additional rules, such as reporting a
207 specific comment under certain conditions, or where the user wishes to report
208 the presence of a variant but not an interpretation (examples in Supplementary
209 Table 1).

210 - *classification_rules.csv* – these rules determine the overall classification profile, based
211 on the combination of resistances in the sequence, for example mono-resistant or
212 multidrug-resistant TB. The defaults are based on the current WHO guidelines
213 (Supplementary Figure 2 and Supplementary File 1).

214 - *db_config.json* – this file provides information about the structure of the mutational
215 catalogue, drugs to include, the values for confidence and resistance levels, and
216 cascade reporting structure (more details in Supplementary File 1)(Figure 1B).

217 tbtAMR provides a default files for each of these accessory files, based on WHO catalogue of
218 mutations version 2[15] and the current WHO guidelines for classification of resistance profile[2].
219 Alternatively, users may choose to modify or replace these files with their own versions to suit
220 their local requirements. As these are simple CSV files, these modifications may be made
221 successfully by non-bioinformaticians, providing flexibility in interpretation and reporting.
222 Note that tbtAMR performance has been investigated based on the default database and
223 interpretation criteria; performance may vary where a bespoke database and/or interpretive
224 criteria are used. The tool has optional additional functionalities, including paired-end read
225 alignment, variant calling and annotation, which require additional dependencies including
226 bwa-mem[17], freebayes[18], snpEff[19], delly[20], bcftools and samtools[21] (Figure 1B). These
227 additional functionalities are implemented in our locally-designed mutAMR tool
228 (https://github.com/MDU-PHL/mutamr). The entire package is available as a conda package.
229

230 **Validation dataset**

231 We included three data sources in our validation dataset (Supplementary Figure 2 and
232 Supplemental File 2). Firstly, we included 2005 sequences generated at Microbiological
233 Diagnostic Unit Public Health Laboratory (MDU PHL) from 2018–2022 and with phenotypic
234 (DST) data generated at the Mycobacterial Reference Laboratory (MRL) (Supplementary
235 Methods). Secondly, 13,777 publicly available sequences were included, selected to be
236 representative of all major global lineages and with phenotypic data available for first-line
237 agents and a subset also having data available Group A, B and Group C drugs[22–25]. Lastly, in
238 order to assess the accurate detection of variants by tbtAMR, we also used simulated genomic
239 data. Paired-end sequence data was generated using TreeToReads software[26–29], directly from
240 the H37rV reference strain (RefSeq accession NC_000962.3) or following introduction of 2–4
241 variants per 10,000 bases at known positions with an error-profile representative of the
242 NextSeq500 instruments at MDU PHL (n= 1830). As resistance testing aims to identify resistant
243 subpopulations of Mtb that may emerge during treatment, these simulated reads were mixed
244 (Supplementary Figure 3) to simulate different allelic frequencies (0.01 to 0.99) across a range
245 of average genome depth (20x to 200x – incremented by 20x).

246 **Measuring the accuracy of Mtb genomic sequence recovery**

247 For simulated genomes, we tested the ability of the tool to correctly identify introduced
248 variants using a minimum depth of 10X and 20X, meaning that to make a base call there was

249 least 10 or 20 reads covering each position. Reads were aligned back to the reference genome
250 and the correctness of base calling by mutAMR was assessed (Supplementary Figure 3).

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

252 We looked at how well inferred phenotype from detected genomic resistance mechanisms
253 (single nucleotide polymorphisms [SNPs], insertions or deletions) correlated with phenotypic
254 DST results. To quantify this, we compared the phenotypes inferred from the presence of
255 SNPs, insertions or deletions in the genome sequences with actual phenotypic susceptibility
256 profiles (Supplementary File 2), and calculated sensitivity, specificity, negative predictive value
257 (NPV), positive predictive value (PPV) and accuracy recorded. When discrepancies between
258 inferred-resistance and the reported phenotypic resistance are identified, these would ideally
259 be resolved with additional phenotypic testing. However, since this was not possible for the
260 discrepancies observed in the publicly available datasets, discrepancy resolution could not be
261 undertaken and any such results were included as discrepancies in performance assessment.
262 tbtAMR also determines phylogenetic lineage, including lineages 1–9 as well as animal
263 adapted species/sub-species[11]. We also assessed the concordance between phylogenetic
264 lineage determined by tbtAMR and the phylogenetic lineage that was supplied as part of the
265 public dataset[25] (Supplementary File 2).

266 **Ethics approval**

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

269 **Role of the funding sources -** The funders played no part in data collection, analysis,
270 interpretation, writing of the manuscript or decision to submit.

271 **Data access** – KAH, TS, NLS, JW and TD had access to data; KAH, BPH, TS and NLS were
272 responsible for the decision to submit the manuscript.

273 Results

274 **Validation of variant detection**

275 *Accuracy of simulated sequence recovery by mutAMR*

276 tbtAMR has the option to align and detect variants in paired-end Illumina WGS data, through
277 mutAMR. We assessed the ability of mutAMR to recover known (introduced) SNPs in
278 simulated sequences, and the minimum average sequence depth for reliable results. Most
279 tools used for detecting variants use a minimum depth criteria of 10X[30,31], meaning that there

must be at least 10 reads aligned to a particular position in the reference genome. However, when we investigated SNPs identified across a range of average sequence depths and allelic frequencies, several false positive (FP) SNPs (mean 6.6, 95% confidence interval (CI) 3.9-9.3) were observed when using the standard criteria of 10X, which may result in false reporting of resistance mechanisms. Increasing the minimum depth for base calling to 20X improved performance, reducing the number of FP SNPs to a mean of 1.1 (CI 0.7-1.5), with little impact on the percentage of introduced SNPs recovered (Figure 2A-B). We further examined the impact of varying average genome depth and allelic frequency on the ability of mutAMR to accurately identify mutations in the simulated sequences. Where average genome depth was less than 40X and allelic frequency was less than 10%, sensitivity of variant identification was low at only 2.1% (CI 1.48%-2.66%) and False Discovery Rate (FDR) was 41.1% (CI 31.1%-51.2%). However, maintaining 20 reads minimum depth for base-calling, an average genome depth of ≥40X and allele frequencies ≥10% gave the best performance (Figure 2C-D), with an overall variant identification sensitivity of 96.3% (CI 95.5%-97.1%) and low FDR of 0.21% (CI 0.11%-0.31%). Detection of other variant types, such as deletions, by mutAMR was consistent with TB-profiler and Mykrobe, using publicly available data. Hence, the minimum acceptable read depth for identification of resistance conferring variants was set to ≥40X and minimum allele frequency of 10%.

Accuracy of phylogenetic lineage calling by tbtAMR

tbtAMR accurately identified lineages compared to lineages reported from public datasets[25], (98.5% concordance 871/884). Of the 13 discordant results (Supplementary Table 2), 12 were cases where tbtAMR identified two different phylogenetic lineages in the sequence. In each case, the reported lineage was one of the lineages detected by tbtAMR. These could reflect mixed sequences or sequences where low coverage may impact base-calling at key sites that differentiate lineages from the reference lineage (lineage 4). It should be noted that the detection of a mixed lineage does not indicate that a sequence is of poor quality for detection of AMR variants.

Inference of phenotypic AMR from genomic data

Many studies have explored the inference of phenotypic AMR from genomic data using various tools[10,24,32,33]. We do not aim to duplicate these high-quality studies in this study, but

312 rather to assess the suitability of tbtAMR for inference and reporting of Mtb AMR to
313 acceptable standards for clinical and public health purposes.
314 We began with a dataset of 15,323 sequences from both publicly available datasets[22–25] and
315 sequences generated in-house using sequencing workflows accredited to ISO standards.
316 Implementing the quality control thresholds and limits of detection outlined above, we
317 excluded 608 sequences based on quality metrics (Supplementary Figure 4), leaving 14,715
318 sequences of sufficient quality to include in the validation of tbtAMR for inference of
319 phenotypic AMR.
320 The performance of tbtAMR in inferring phenotypic resistance to first-line drugs was excellent,
321 with high performance across all first-line drugs (Table 2 and Figure 3) and was consistent with
322 the performance of both TB-profiler and Mykrobe (Supplementary Figure 5). Lower positive
323 predictive values for ethambutol (81.1% CI 79.4%-82.7%) were due to detection of variants in
324 *embB* at codon 360 where phenotype reported was susceptible. This observation is likely due
325 to known challenges in consistency of ethambutol DST and are consistent with other
326 studies[10,24,32,33]. Prediction of pyrazinamide resistance also has lower sensitivity than other
327 first-line drugs (80.0% CI 75.4%-84.0%) and is likely due both challenges in pyrazinamide DST
328 and less robust understanding of the molecular mechanisms leading to pyrazinamide
329 resistance[5].
330 tbtAMR predicted resistance to groups A, B and C (Table 1) with performance comparable to
331 other studies[10,24,32,33] (Supplementary Figure). The high number of false-negative and false-
332 positive results for prediction of phenotypic resistance (Table 2) are likely to be due to
333 incomplete understanding of the mechanisms of resistance to these drugs, global paucity of
334 data (phenotypic and genotypic) and challenging or inconsistent phenotypic testing for some
335 drugs. Although tbtAMR performance was consistent with other tools and use of updated
336 catalogue improved performance compared on the previous WHO catalogue, prediction of
337 bedaqualine, delaminid, linezolid and clofazimine was not of sufficient performance to allow
338 for routine reporting of resistance to these drugs from genomic data in a CPHL setting, due to
339 the high rates of both false positive and false negative results (Supplementary Figure 6).
340 **Implementation process**
341 As AMR genotypes and inferred antibiograms for Mtb were new to most clinicians, additional
342 interpretive comments were created for inclusion in reports – for example, explaining the
343 difference between confidence level and level of resistance in the inferred phenotype

344 (Supplementary Methods and Supplementary Files 3). Clinical and laboratory colleagues were
345 consulted about the draft report design to ensure the formatting and explanations were clear
346 and unambiguous in our local setting. Once validation and report design were complete, Mtb
347 genomic AMR inference using the tbtAMR tool was implemented into existing genomic
348 workflows, including standard operating procedure documentation (SOP), staff training,
349 incorporation into the laboratory information management system, and testing of reporting
350 and feedback loops (e.g. reporting of samples that failed QC).
351 To address any discrepancies in phenotypic and genotypic results, a multidisciplinary expert
352 panel for Mtb AMR was formed, meeting monthly to discuss individual cases and provide
353 expert recommendations to clinicians where discrepancies were identified. This provides the
354 opportunity for ongoing dialogue between the wet-lab microbiologists, clinicians,
355 epidemiologists, medical microbiologists and bioinformaticians to monitor for AMR
356 determinants of interest, discuss individual cases and discrepancies, and undertake
357 prospective validation of novel or uncharacterised AMR determinants.
358 In preparation for ISO-equivalent accreditation, revalidation and reverification processes were
359 also defined for tbtAMR (Supplementary Methods). Subsequently, the Mtb genomic AMR
360 inference workflow has now been accredited to ISO 15189:2022 standards.

361 ## Discussion

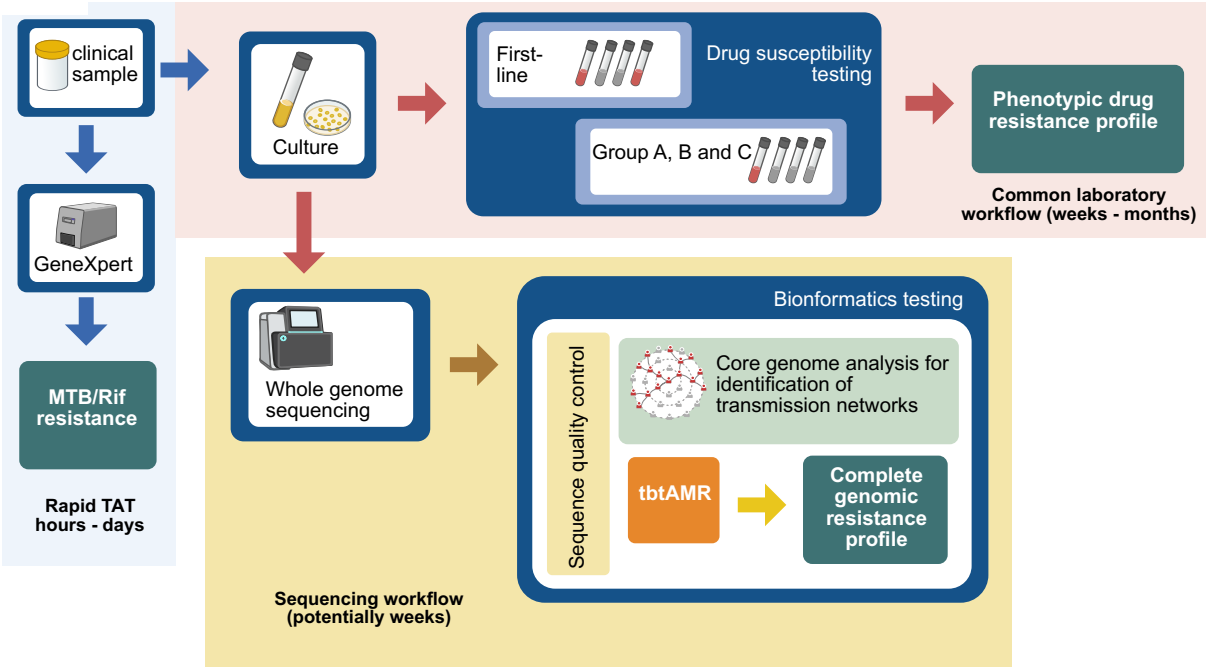
362 We have reported here the development of a custom Mtb AMR analysis and reporting pipeline
363 for use in the clinical and public health microbiology setting, with easily customisable
364 interpretative and classification criteria to maximise PPV and NPV for prediction of AMR in
365 Mtb. Performance of inferred AMR using our process is consistent with the performance of
366 other tools[10,33] (Supplementary Figure 5). This pipeline has been robustly validated,
367 implemented and accredited to ISO standards in our laboratory, with reverification strategies
368 for ongoing improvement, and reporting processes put in place.
369 Globally, we have seen increasing availability of WGS in clinical and public health labs,
370 particularly since the COVID pandemic, resulting in a massive increase in sequencing capacity
371 and data[34]. However, significant skills gaps exist in the CPHL workforce[33,35,36], particularly in
372 LMICs, limiting the ability to use, develop and/or maintain open-source software, and
373 proprietary alternatives may be expensive. Whilst open-source software is accessible and free
374 to users, it can be challenging to implement in a CPHM laboratory setting. These tools are
375 mostly developed and maintained by independent researchers, and may not always be
376 maintained or frequently updated, may be difficult to install, or not meet the needs of the
377 CPHM lab[37]. Maintaining and implementing such tools in an ISO-accredited laboratory
378 structure can be time-consuming and difficult, requiring significant staff time and resources[9],
379 as laboratories cannot be selective about the updates adopted if the tool is maintained
380 elsewhere, issues which are further exacerbated in resource-limited settings[38]. tbtAMR and
381 its supporting documentation aims to address some of these issues for CPHM, by providing
382 validation methods and datasets, reverification processes and example reports. Additionally,
383 due to minimal additional dependencies, tbtAMR can easily be installed and run on a laptop
384 or be incorporated into large and complex bioinformatics workflows easily.
385 tbtAMR is designed to be flexible, allowing users to choose default settings and accessory files,
386 or using user-supplied criteria which can be tailored as required for local needs. These files
387 are designed to be modifiable by a non-developer (such as a genomics-literate medical
388 microbiologist or scientist), without the requirement for coding skills. This allows users to
389 modify interpretations based on local needs – for example, in a high-TB-burden setting, the
390 threshold for reporting resistance may be lower than a low-TB-burden setting, and as such,
391 the user may choose a lower threshold to report resistance (e.g., report all low-level resistant

392 mutations as resistant). Alternatively, cascade reporting rules may be modified to suit the TB
393 drugs available in different settings. In both situations, we believe it is best for the user to be
394 able to set these parameters, rather than relying on the judgement of the software developer
395 to determine what is 'best'.
396 AMR determinants are often reported as a binary 'present/absent' variable, inferring a
397 'resistant' or 'susceptible' phenotype respectively, but this does not always accurately reflect
398 the complexity of genotype-phenotype correlations. It is important to ensure that mutations
399 used to infer resistance have solid evidence supporting a role as genomic determinants of
400 resistance. The new WHO catalogue and documentation provide some information for
401 mutations which are likely to confer 'borderline' resistance such as the rpoB His445Asn
402 variant[15] and confidence levels which may be used to aid interpretation. The structure of
403 tbtAMR allows us to provide meaningful reports to clinicians (based on high confidence
404 mutations), capturing the complexity of genotype-phenotype relationships, whilst still being
405 able to prospectively monitor and validate additional mutations (mutations from all
406 confidence levels). This is particularly relevant for the newer second-line drugs including
407 bedaquiline, delaminid and linezolid, where a paucity of data (locally and globally) still limits
408 our ability to adequately validate inference of resistance to these drugs for reporting in a CPHL
409 setting. Even for drugs which are well studied, discrepancies can still occur, and further
410 research and iterative validation will only improve the performance of tbtAMR and other tools
411 used by Mtb genomics community, ultimately leading to enhanced patient outcomes.
412 Performing gDST for Mtb in CPHM settings is a balancing act, weighing direct impacts on
413 patient management on one hand, and pragmatic laboratory management with finite
414 resources in the other. Sequencing processes in a CPHL laboratory are usually dictated by
415 factors such as costs and efficiency. Thus, it is not usually possible for CPHM labs to routinely
416 obtain the sequence depth required to reliably identify high confidence variants below 10%
417 allelic frequency[39]. Furthermore, at lower depth of coverage identification of false positive
418 variants[40] (Figure 2) could lead to erroneous reporting of gDST. The default settings used in
419 tbtAMR aim to address these considerations, particularly for a CPHM setting, either in high-
420 income country or LMIC. However, these settings are not fixed and may be modified for
421 settings with differing cost and capacity considerations, including research settings.
422 tbtAMR represents a flexible yet robust and accurate pipeline that leverages the validation
423 undertaken here and other quality studies[22,24,32,33] to achieve the best predictive power for

424 patient management, and simplified outputs for clinical and public health microbiology.
425 Additionally, the tbtAMR tool has also been implemented with a graphic user interface (GUI)
426 as part of the Centre for Pathogen Genomics (CPG) bioinformatics portal
427 (https://portal.cpg.unimelb.edu.au), which provides access to bioinformatics tools for LMICs
428 and others in resource-limited settings. Whilst this does not alleviate the need for medical
429 microbiologists and scientists in resource-limited settings to develop genomic literacy to be
430 able to optimally use tbtAMR and its results, we hope that providing an accessible GUI with
431 relevant documentation will at least open the door for high TB-burden countries to access our
432 tools.
433 Our results also highlight areas where further research can enhance our knowledge of AMR
434 determinants in Mtb, with the correlation between AMR determinants and phenotypes with
435 new second-line Mtb drugs being a key priority. Further enhancements of diagnostic
436 processes, such as sequencing directly from samples, will allow us to maximise the benefit of
437 genomic technologies for patient management. Ongoing engagement with stakeholders,
438 including researchers, clinicians and public health officials, ensures that the processes
439 established can be improved and refined where needed to continue to improve patient
440 management and inform timely public health responses.

441 **Figures**

A


clinical sample
GeneXpert
MTB/Rif resistance
Rapid TAT hours - days
Culture
First-line
Drug susceptibility testing
Group A, B and C
Phenotypic drug resistance profile
Common laboratory workflow (weeks - months)
Whole genome sequencing
Sequencing workflow (potentially weeks)
Bionformatics testing
Sequence quality control
Core genome analysis for identification of transmission networks
tbtAMR
Complete genomic resistance profile

442

B

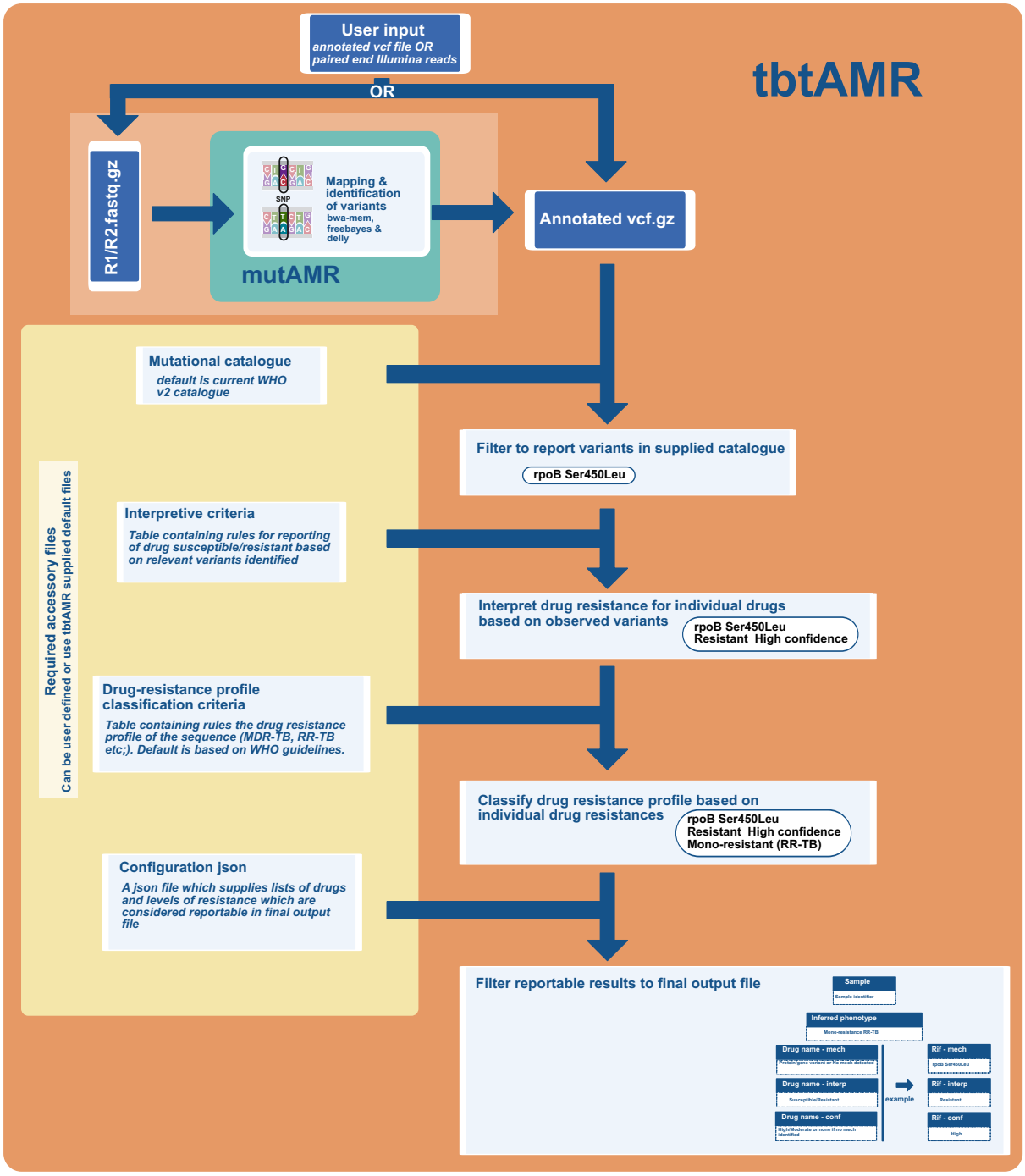


Figure 1 – Incorporation of tbtAMR into *M. tuberculosis* diagnosis and testing workflow in a reference laboratory.

A. Diagnostic testing pathways for Mtb: *M. tuberculosis* and potential rifampicin resistance can be identified rapidly using GeneXpert (closed-system PCR for most common rifampicin resistance mutations) in a diagnostic laboratory (turnaround time hours–days). Traditional culture methods require samples to be inoculated to solid media (agar slopes) and/or liquid media (most commonly MGIT). Cultures may take up to 6-8 weeks to flag positive. From positive culture, phenotypic drug susceptibility testing is then commenced, taking up to two weeks to be completed, or longer if cascade testing is used (Group A, B and C drugs only set up when resistance to first-line drugs is identified). Total turnaround times from sample to culture and phenotypic DST results is usually 6 weeks-3 months. If optimised, whole genome sequencing can potentially reduce this turnaround time and provide a comprehensive

resistance profile without the need for further testing, whilst also providing important contributions to public health control efforts through contact tracing. **B. tbtAMR pipeline:** tbtAMR inputs can be either an annotated variant file (vcf format, from short-read or long-read sequences) based on H37rV v3 reference genome or paired-end short-read data (fastq file), making tbtAMR sequencing platform agnostic. tbtAMR requires 4 accessory files, defaults are supplied with tbtAMR, but users can their own if appropriate. By default tbtAMR uses version 2 of the WHO mutational catalogue as default with accompanying files containing criteria for interpretation, classification and reporting. The accessory files are the mechanism by which tbtAMR provides interpretation of detected variants, performs classification of drug resistance profile and filters results for reporting in final output file.

Abbreviations: Mtb, *Mycobacterium tuberculosis*; Rif, rifampicin; TAT, turnaround time. Variant nomenclature example: “rpoB_Ser450Leu” denotes a change in the amino acid from serine to leucine at position 450 in the RpoB protein sequence.

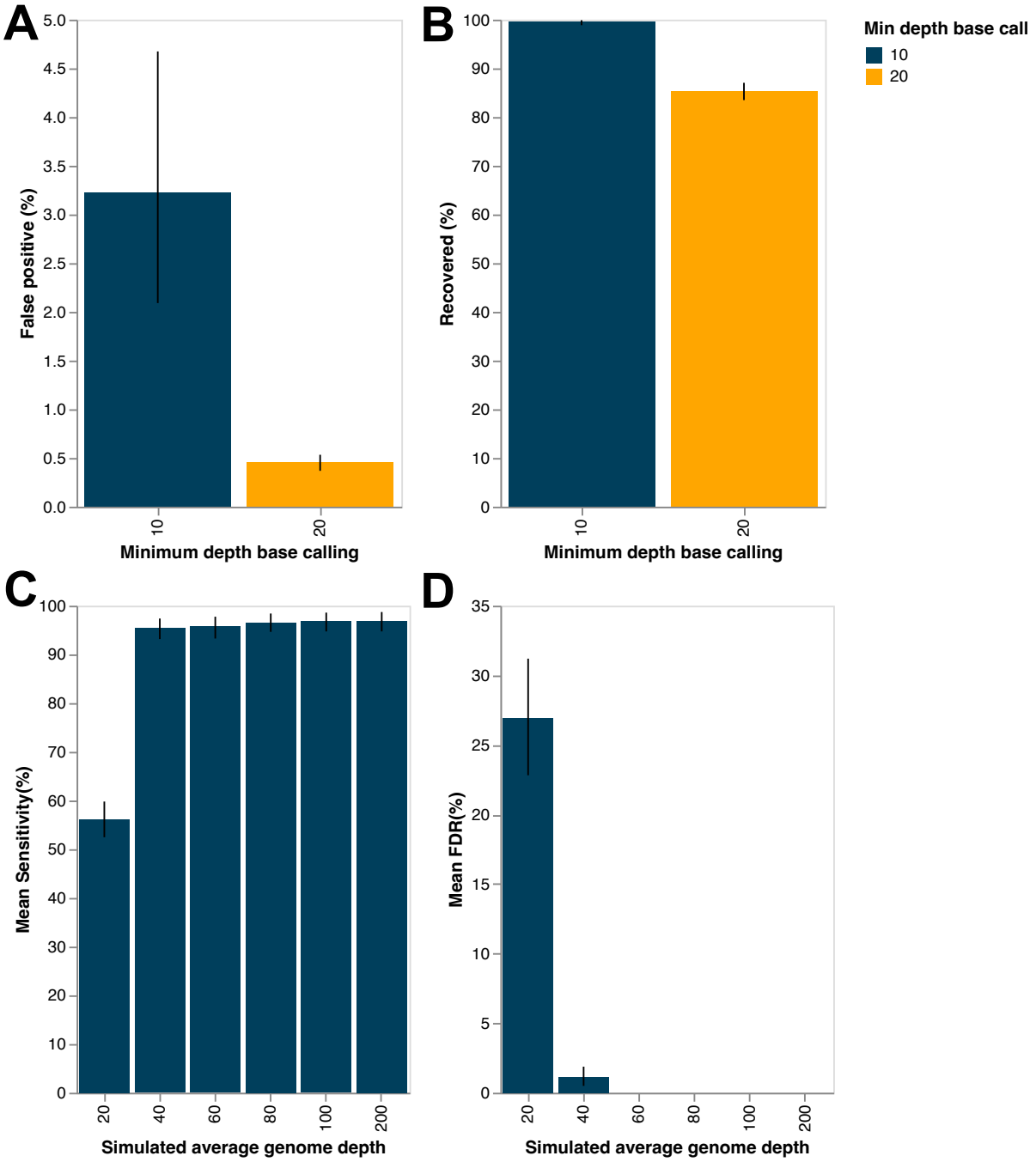
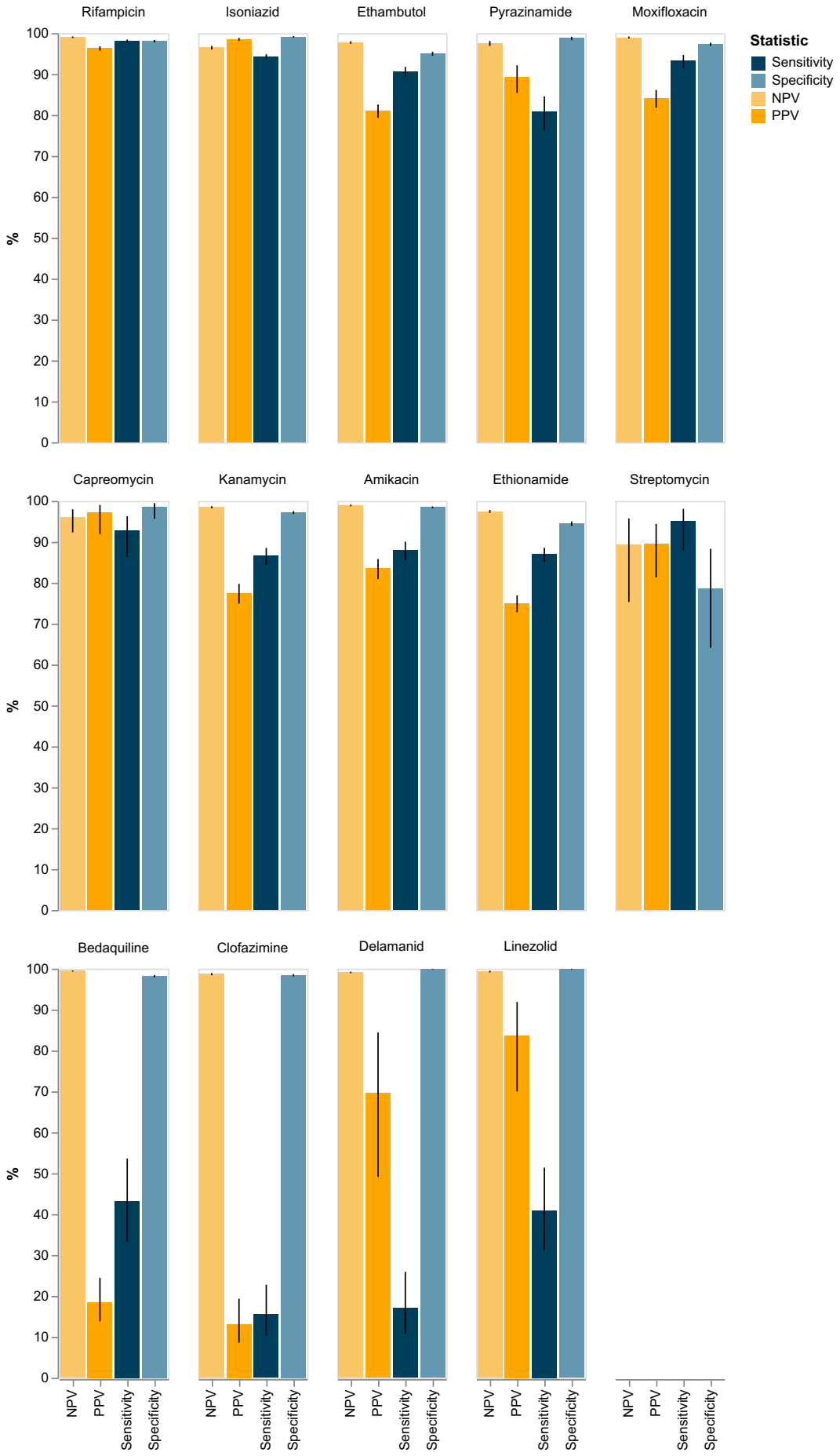


Figure 2 – Performance of mutAMR to recover SNPs from Mtb sequences

To assess the performance of mutAMR, implemented by tbtAMR to accurately recover mutations in Mtb sequences, simulated genome data across a range of average genome depths from 10X to 200X at allelic frequencies 10-100% were assessed. The impact of minimum read depth for base calling at an individual position in the genome (depth 10 or 20) was assessed for: the percentage of false positive SNP calls (FP) identified (**Panel A**) and the percentage of introduced SNPs recovered by mutAMR (**Panel B**). Using a minimum-depth criteria of 20X for individual base calling, **C.** SNP calling sensitivity and **D.** false discovery rate (FDR) were assessed average genome depth using average genome depth of coverage of ≥40X and allelic frequency ≥10% (error bars indicate 95% confidence interval).


Rifampicin
Isoniazid
Ethambutol
Pyrazinamide
Moxifloxacin
Capreomycin
Kanamycin
Amikacin
Ethionamide
Streptomycin
Bedaquiline
Clofazimine
Delamanid
Linezolid
%
0
10
20
30
40
50
60
70
80
90
100
NPV
PPV
Sensitivity
Specificity
Statistic
Sensitivity
Specificity
NPV
PPV

481

482 **Figure 3 – Performance of tbtAMR inference of phenotypic susceptibility**
483 Inferred phenotypes predicted by the tbtAMR pipeline were compared to phenotypic DST
484 data, true positive (TP) was recorded when a mutation was reported and the phenotype was
485 resistant; a true negative (TN) was recorded when no mutation was reported and the
486 phenotype was susceptible; a false positive (FP) was recorded when a mutation was reported,
487 but the phenotype was susceptible, and a false negative (FN) was recorded when no mutation
488 was reported, and the phenotype was resistant. Sensitivity, specificity, positive and negative
489 predictive values calculated and expressed as a percentage (error bars indicate 95%
490 confidence intervals). DST, direct susceptibility testing; PPV, positive predictive value; NPV,
491 negative predictive value.
492

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; the diagnostic laboratories who diligently provide samples for testing; and the Victorian TB Program, and Department of Health Victoria who fund these services and engage enthusiastically with the laboratories. We also thank the developers and maintainers of the open-source software used and cited in this publication. This work was funded by the Australian National Health and Medical Research Council (NHMRC), Medical Research Futures Fund (FSPGN00049), and Investigator Grant (GNT1196103) to BPH.

Data sharing

Raw sequence data generated at MDU PHL has been uploaded to the Sequence Read Archive under BioProject PRJNA857537. Accessions for all sequences generated and deposited in public repositories can be found in Supplemental File 1.

All results used for interpretation and generation of results, including phenotypic DST results and genomic results used in this study are available in Supplemental File 1 and readily available on GitHub. Further instructions for installing and running tbtamr as well as the complete code base are also available on GitHub (https://github.com/MDU-PHL/tbtamr).

Author Contributions

Kristy Horan – developed software, study design, data analysis, manuscript writing and editing.

Linda Viberg – performed DNA extractions, reviewed data, edited manuscript

Susan Ballard – contributed to study design, laboratory supervision, edited manuscript

Maria Globan – laboratory supervision, reviewed data, edited manuscript

Wytamma Wirth – developed software, edited manuscript

Katherine Bond – study design, laboratory supervision, edited manuscript

Jessica R Webb – created figures, edited manuscript

Thinley Dorji – created figures, edited manuscript

Deborah Williamson – contributed to study design, edited manuscript

Michelle Sait – performed genomic sequencing, edited manuscript

Ee Laine Tay – reviewed data, edited manuscript

Justin T Denholm – contributed to study design, edited manuscript

Torsten Seemann – study design, software development, validation protocols, edited manuscript

Benjamin P Howden – funding, laboratory supervision, contributed to study design, edited manuscript

Norelle L Sherry – study design, contributed to software development, reporting templates, data analysis, manuscript writing and editing.

References

1 Bennett J, Dolin R, Blaser MJ. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 9th edn. .

2 WHO Consolidated Guidelines on Tuberculosis, Module 4: Treatment - Drug-Resistant Tuberculosis Treatment. https://www.who.int/publications-detail-redirect/9789240007048 (accessed March 5, 2022).

3 WHO consolidated guidelines on tuberculosis. Module 4: treatment - drug-susceptible tuberculosis treatment. Geneva: World Health Organization; https://iris.who.int/bitstream/handle/10665/353829/9789240048126-eng.pdf?sequence=1 (accessed Oct 1, 2024).

4 TB guidelines. https://www.who.int/publications/digital/global-tuberculosis-report-2021/featured-topics/tb-guidelines (accessed March 5, 2022).

5 Carrol K, Pfaller M, Landry M. Manual of Clinical Microbiology, 12th edn. Wiley, 2019.

6 Denholm JT, de Vries G, Anthony R, *et al.* Considering best practice standards for routine whole-genome sequencing for TB care and control. *IJTLD Open* 2024; **1**: 431–6.

7 Donnan EJ, Marais BJ, Coulter C, *et al.* The use of whole genome sequencing for tuberculosis public health activities in Australia: a joint statement of the National Tuberculosis Advisory Committee and Communicable Diseases Genomics Network. *Commun Dis Intell (2018)* 2023; **47**. DOI:10.33321/cdi.2023.47.8.

8 14:00-17:00. ISO 15189:2022. ISO. https://www.iso.org/standard/76677.html (accessed July 4, 2023).

9 Ballard SA, Sherry NL, Howden BP. Public health implementation of pathogen genomics: the role for accreditation and application of ISO standards. *Microbial Genomics* 2023; **9**: 001097.

10 Hunt M, Bradley P, Lapierre SG, *et al.* Antibiotic resistance prediction for Mycobacterium tuberculosis from genome sequence data with Mykrobe. *Wellcome Open Res* 2019; **4**: 191.

11 Phelan JE, O'Sullivan DM, Machado D, *et al.* Integrating informatics tools and portable sequencing technology for rapid detection of resistance to anti-tuberculous drugs. *Genome Medicine* 2019; **11**: 41.

12 ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads | Microbiology Society. https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000131 (accessed July 4, 2023).

13 Spies R, Crook DW, Peto TEA, *et al.* A Systematic Evaluation of Automated Mycobacterium Tuberculosis Complex Whole Genome Sequencing Analysis Pipelines. 2024; published online Dec 23. DOI:10.2139/ssrn.5064085.

14 Walker TM, Miotto P, Köser CU, *et al.* The 2021 WHO catalogue of Mycobacterium tuberculosis complex mutations associated with drug resistance: a genotypic analysis. *The Lancet Microbe* 2022; **3**: e265–73.

15 Catalogue of mutations in Mycobacterium tuberculosis complex and their association with drug resistance. https://www.who.int/publications-detail-redirect/9789240028173 (accessed Nov 23, 2022).

16 MDU-PHL/tbtamr. 2024; published online Oct 23. https://github.com/MDU-PHL/tbtamr (accessed Nov 12, 2024).

17 Vasimuddin Md, Misra S, Li H, Aluru S. Efficient Architecture-Aware Acceleration of BWA-MEM for Multicore Systems. In: 2019 IEEE International Parallel and Distributed Processing Symposium (IPDPS). 2019: 314–24.

18 Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. 2012; published online July 20. http://arxiv.org/abs/1207.3907 (accessed Oct 1, 2024).
19 Cingolani P, Platts A, Wang LL, *et al.* A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w ^{1118} ; iso-2; iso-3. *Fly* 2012; **6**: 80–92.
20 DELLY: structural variant discovery by integrated paired-end and split-read analysis | Bioinformatics | Oxford Academic. https://academic.oup.com/bioinformatics/article/28/18/i333/245403?login=false (accessed Oct 1, 2024).
21 Danecek P, Bonfield JK, Liddle J, *et al.* Twelve years of SAMtools and BCFtools. *GigaScience* 2021; **10**: giab008.
22 Quantitative measurement of antibiotic resistance in Mycobacterium tuberculosis reveals genetic determinants of resistance and susceptibility in a target gene approach. *Nat Commun* 2024; **15**: 488.
23 Ezewudo M, Borens A, Chiner-Oms Á, *et al.* Integrating standardized whole genome sequence analysis with a global Mycobacterium tuberculosis antibiotic resistance knowledgebase. *Sci Rep* 2018; **8**: 15382.
24 Coll F, Phelan J, Hill-Cawthorne GA, *et al.* Genome-wide analysis of multi- and extensively drug-resistant Mycobacterium tuberculosis. *Nat Genet* 2018; **50**: 307–16.
25 Prediction of Susceptibility to First-Line Tuberculosis Drugs by DNA Sequencing. *New England Journal of Medicine* 2018; **379**: 1403–15.
26 Huang W, Li L, Myers JR, Marth GT. ART: a next-generation sequencing read simulator. *Bioinformatics* 2012; **28**: 593–4.
27 McTavish EJ. TreeToReads. 2022; published online Sept 14. https://github.com/snacktavish/TreeToReads (accessed Nov 16, 2022).
28 Rambaut A, Grassly NC. Seq-Gen: an application for the Monte Carlo simulation of DNA sequence evolution along phylogenetic trees. *Comput Appl Biosci* 1997; **13**: 235–8.
29 Sukumaran J, Holder MT. DendroPy: a Python library for phylogenetic computing. *Bioinformatics* 2010; **26**: 1569–71.
30 Seemann T. Snippy. 2022; published online March 19. https://github.com/tseemann/snippy (accessed March 26, 2022).
31 Phelan J. jodyphelan/TBProfiler. 2022; published online Feb 15. https://github.com/jodyphelan/TBProfiler (accessed Feb 28, 2022).
32 Shaw B, von Bredow B, Tsan A, Garner O, Yang S. Clinical Whole-Genome Sequencing Assay for Rapid Mycobacterium tuberculosis Complex First-Line Drug Susceptibility Testing and Phylogenetic Relatedness Analysis. *Microorganisms* 2023; **11**: 2538.
33 Mahé P, El Azami M, Barlas P, Tournoud M. A large scale evaluation of TBProfiler and Mykrobe for antibiotic resistance prediction in Mycobacterium tuberculosis. *PeerJ* 2019; **7**: e6857.
34 Black A, MacCannell DR, Sibley TR, Bedford T. Ten recommendations for supporting open pathogen genomic analysis in public health. *Nat Med* 2020; **26**: 832–41.
35 Sundermann AJ, Rosa R, Harris PNA, *et al.* Pathogen genomics in healthcare: overcoming barriers to proactive surveillance. *Antimicrobial Agents and Chemotherapy* 2024; **69**: e01479-24.
36 Pronyk PM, de Alwis R, Rockett R, *et al.* Advancing pathogen genomics in resource-limited settings. *Cell Genomics* 2023; **3**: 100443.
37 Siepel A. Challenges in funding and developing genomic software: roots and remedies. *Genome Biology* 2019; **20**: 147.

38 Helmy M, Awad M, Mosa KA. Limited resources of genome sequencing in developing countries: Challenges and solutions. *Applied & Translational Genomics* 2016; **9**: 15–9.

39 Goossens SN, Heupink TH, De Vos E, *et al.* Detection of minor variants in Mycobacterium tuberculosis whole genome sequencing data. *Briefings in Bioinformatics* 2022; **23**: bbab541.

40 Seah YM, Stewart MK, Hoogestraat D, *et al.* In Silico Evaluation of Variant Calling Methods for Bacterial Whole-Genome Sequencing Assays. *Journal of Clinical Microbiology* 2023; **61**: e01842.