In silico evaluation of WHO-endorsed molecular methods to detect drug resistant tuberculosis

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Universal drug susceptibility testing (DST) for tuberculosis is a major goal of the END TB strategy. PCR-based molecular diagnostic tests have been instrumental in increasing DST globally and several assays have now been endorsed by the World Health Organization (WHO) for use in the diagnosis of drug resistance. These endorsed assays, however, each interrogate a limited number of mutations associated with resistance, potentially limiting their sensitivity compared to sequencing-based methods. We applied an in silico method to compare the sensitivity and specificity of WHO-endorsed molecular based diagnostics to the mutation set identified by the WHO mutations catalogue using phenotypic DST as the reference. We found that, in silico, the mutation sets used by probe-based molecular diagnostic tests to identify rifampicin, isoniazid, pyrazinamide, levofloxacin, moxifloxacin, amikacin, capreomycin and kanamycin resistance produced similar sensitivities and specificities to the WHO mutation catalogue. PCR-based diagnostic tests were most sensitive for drugs where mechanisms of resistance are well established and localised to small genetic regions or a few prevalent mutations. Approaches using sequencing technologies can provide advantages for drugs where our knowledge of resistance is limited, or where complex resistance signatures exist.

In 2020, an estimated 10 million people became ill with tuberculosis (TB), and of these 1.5 million died from the disease1. Mycobacterium tuberculosis with antibiotic resistance poses a more severe threat; treatment success for patients with rifampicin-resistant (RR) or multidrug-resistant (MDR) TB (resistant to the most effective first-line drugs, rifampicin (RIF) and isoniazid (INH)) stands at 59% worldwide1. Resistance to second-line drugs used to treat MDR infections is also of concern as extensively drug-resistant (XDR) TB infections (RR/MDR plus resistance to fluoroquinolones and either bedaquiline or linezolid) can potentially develop, leaving patients with few treatment options. In order to reduce TB incidence by 80% and deaths by 90%2, the World Health Organisation (WHO) has identified universal drug susceptibility testing (DST) as a key component of the END TB strategy2,3. Universal DST is important to ensure that patients are placed on the most effective treatment regimens and to identify drug resistant strains and prevent their further spread.

Molecular diagnostic tests offer a simpler and more rapid solution to detect drug resistance in comparison to time-consuming phenotypic methods, furthermore laboratory testing is not always possible in resource limited settings due to infrastructure and trained personnel requirements4. M. tuberculosis lends itself to detection by molecular diagnostic tools because the majority of drug resistance for the most commonly used anti-TB drugs can be attributed to well characterized genetic mutations including single nucleotide polymorphisms (SNPs) and insertions or deletions5.

Among the several molecular diagnostic tests developed and approved by the WHO6 to date, GeneXpert MTB/RIF is the most widely used globally for TB and RIF resistance detection and has facilitated increased testing and RIF resistance surveillance in regions with a high TB burden7,8. However, individual molecular diagnostic tests are not designed to include exhaustive lists of resistance associated mutations. For example, the rpoB I491F mutation underlying an MDR outbreak in Eswatini was not detected by GeneXpert MTB/RIF9 which may have contributed to spread of undetected RIF resistance in South Africa10. Current molecular diagnostic tests can also give false positive resistance diagnosis, which could result in the unnecessary, longer treatment regimens.

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including more toxic antitubercular drugs. For instance, a mutation that does not confer resistance, gyrA A90G, prevents hybridization of a wild-type probe in the Hain Genotype MTBDRsl v1 and v2 assays resulting in a misleading test interpretation of fluoroquinolone resistance\(^1\)\(^2\).

Whole genome sequencing (WGS) offers an alternative to current molecular diagnostic testing; it provides a comprehensive report of the genetic variation present in a *M. tuberculosis* isolate and can be used to successfully predict resistance and susceptibility to antibiotics in both TB \(^13\)\(^-\)\(^15\) and other infectious diseases. \(^16\)\(^-\)\(^17\) This method of detecting SNPs associated with resistance is already being widely used for comprehensive DST in some high-income countries, including the United Kingdom where all *M. tuberculosis* isolates now undergo WGS. However, the current WGS workflow still has time and infrastructure limitations as *M. tuberculosis* bacilli must be isolated and cultured from the clinical sample prior to sequencing to ensure sufficient coverage of the bacterial genome. \(^18\)

Targeted next generation sequencing (tNGS) could be a more appropriate solution for resource limited settings as it can be implemented directly from clinical samples, offering a more rapid resistance diagnosis, and performs comparably to WGS platforms for detecting resistance conferring mutations. \(^19\)\(^-\)\(^22\)

Ultimately, the success of both sequencing and molecular diagnostic tests for TB resistance prediction relies on the production of a comprehensive catalogue of TB genetic mutations. This need has led to the collection of large, global, matched genotypic and phenotypic datasets, from which statistical tests, machine learning or genome wide association studies can be used to identify the mutations that are associated and not associated with resistance. \(^23\) Most recently, the WHO published a catalogue of over 17,000 mutations associated with resistance to 13 anti-TB drugs using data from over 38,000 *M. tuberculosis* isolates using a standardized statistical approach. \(^15\)\(^-\)\(^24\) The catalogue is therefore recommended for the standardized interpretation of sequencing based DST approaches and the mutations identified can also be used to inform the development of novel molecular diagnostic tests.

Although next generation sequencing (NGS) approaches offer an attractive solution to some of the problems encountered when using molecular diagnostic tests, sequencing approaches are not yet implementable in all settings due to the infrastructure requirements. It is therefore important to evaluate how well different molecular tests could detect resistance by the mutations that they cover, and how the performance compares to catalogue-based resistance prediction. An in silico evaluation method has particular advantages as it can quickly evaluate proposed lists of mutations for identifying resistance in new or revised drug resistance assays in future. In this study, we measure the in silico performance of WHO endorsed molecular assays, based solely on the mutations they cover, in predicting drug resistance within the globally diverse dataset used to derive the WHO catalogue, comparing it to the performance of the mutations from the WHO catalogue itself, using phenotypic DST as the reference standard.

**Methods**

**Compilation of mutations.** For our exhaustive list of resistance associated mutations, we used the WHO 2021 catalogue and included mutations that were categorised as (1) associated with resistance or (2) associated with resistance interim. \(^15\)\(^-\)\(^24\) We evaluated WHO endorsed molecular diagnostic tests that detected resistance to one or more of the following drugs: rifampicin, isoniazid, pyrazinamide, ethionamide, levofloxacin, moxifloxacin, amikacin, capreomycin or kanamycin. Where documented, specific mutations detected by mutant probes and regions where any mutation would result in disruption of binding of a wild-type probe were identified based on package inserts or literature (Table 1). Specific probes used for detecting mutations not associated with resistance, or positions in regions that wild type probes are agnostic to were also included where available. If a list of specific probes or regions was not available for any molecular diagnostic assay, secondary evidence from literature was used. The list of mutations detected by each diagnostic test for use in in silico evaluations of the tests is summarised in Table 1.

**Dataset and filtering.** A total of 38,126 isolates used to compile the 2021 WHO catalogue of *M. tuberculosis* complex mutations associated with drug resistance with phenotypic and WGS data for either rifampicin, isoniazid, pyrazinamide, ethionamide, levofloxacin, moxifloxacin, amikacin, capreomycin or kanamycin were considered for this analysis. \(^15\)\(^-\)\(^24\) Isolates with known resistance conferring mutations identified via sequencing and a susceptible phenotype for the corresponding drug were not removed from this sample set; although these samples may have been mislabelled, they were kept in the dataset to reflect real world performance, where there will be a degree of mislabelling and phenotypic error. The WGS data that we used was processed for the compilation of the 2021 WHO catalogue, and as such, nucleotide positions where the bioinformatic pipeline used reported no data or where there was equal support for two different nucleotides (null call) and positions where there was evidence for multiple nucleotides or where there was insufficient coverage (filter-fail) were assumed wild-type. Therefore, heterozygous calls (fraction of read support < 90%) were not considered for this analysis, although we note that these could be picked up by WGS and catalogue-based prediction pipelines, as well as some molecular diagnostic tests. \(^45\)

For this analysis we excluded phenotypic data for drugs where one or more of the corresponding genes interrogated by either the catalogue or a molecular test (Table S1) had an excessive number of filter-fail or null calls for the variants identified, on the assumption that the genetic data were unreliable. The definition used for ‘excessive’ was the probability of that many filter fail or null calls being less than 1%, assuming a Poisson distribution. \(^15\)\(^-\)\(^24\) Upon application of these filtering criteria, 38,111 isolates remained for further analysis; a breakdown of the number of isolates with phenotypic and genetic information for each drug is presented in Table 2. The origin of the 38,111 samples in the final set is shown in Table S2.

**In silico performance of sequencing and molecular diagnostic tests.** To evaluate the in silico sensitivity and specificity of sequencing compared to phenotypic DST as reference, we examined the proportion of...
| Test                                      | Drug          | Resistant mutant probes | Wild type probes | Susceptible probes/agnostic | References |
|------------------------------------------|---------------|-------------------------|------------------|-----------------------------|------------|
| Abbott RealTime MTB RIF/INH             | Rifampicin    | rpoB 426:452            | katG 315         |                              | 6,25,26    |
|                                          | Isoniazid     | katG S315              | inha c-15t       |                              | 6,25,26    |
|                                          | Ethionamide   | inha c-15t, t-8a, t-8c |                  |                              | 6,41–43    |
|                                          | Fluoroquinolones| gyrA A90V, S91P, D94A, D94N/Y, D94G, D94H | gyrA 85:96 | gyrA S95T | 6,25,26 |
|                                          | Ethionamide   | inha c-15t, t-8a, t-8c |                  |                              | 6,41–43    |
|                                          | Fluoroquinolones| gyrA A90V, S91P, D94A, D94N/Y, D94G, D94H | gyrA 85:96 | gyrA S95T | 6,25,26 |
|                                          | Ethionamide   | inha c-15t, t-8a, t-8c |                  |                              | 6,41–43    |
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|                                          | Ethionamide   | inha c-15t, t-8a, t-8c |                  |                              | 6,41–43    |
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|                                          | Ethionamide   | inha c-15t, t-8a, t-8c |                  |                              | 6,41–43    |
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|                                          | Ethionamide   | inha c-15t, t-8a, t-8c |                  |                              | 6,41–43    |
|                                          | Fluoroquinolones| gyrA A90V, S91P, D94A, D94N/Y, D94G, D94H | gyrA 85:96 | gyrA S95T | 6,25,26 |
|                                          | Ethionamide   | inha c-15t, t-8a, t-8c |                  |                              | 6,41–43    |
|                                          | Fluoroquinolones| gyrA A90V, S91P, D94A, D94N/Y, D94G, D94H | gyrA 85:96 | gyrA S95T | 6,25,26 |
|                                          | Ethionamide   | inha c-15t, t-8a, t-8c |                  |                              | 6,41–43    |
|                                          | Fluoroquinolones| gyrA A90V, S91P, D94A, D94N/Y, D94G, D94H | gyrA 85:96 | gyrA S95T | 6,25,26 |
|                                          | Ethionamide   | inha c-15t, t-8a, t-8c |                  |                              | 6,41–43    |
|                                          | Fluoroquinolones| gyrA A90V, S91P, D94A, D94N/Y, D94G, D94H | gyrA 85:96 | gyrA S95T | 6,25,26 |

Table 1. Mutations detected by WHO-endorsed molecular diagnostic tests. Regions are denoted by ‘:’ and are inclusive. Positions relating to codons rather than nucleotide positions are in bold. Amino acids are denoted by upper case letters and nucleotides by lower case. Promoter mutations are denoted by a ‘–’. The eis c-14t probe is included for aminoglycoside resistance detection by GenoType MTBDRsl v2 as the mutation is included in the WHO 2021 catalogue as associated with resistance to these drugs.
Compilation of clinical performance of molecular diagnostic tests. Where possible, we used pooled clinical sensitivity and specificity data from systematic reviews of tests to indicate the likely performance of the molecular diagnostic tests for in silico analyses. Where this information was not available, we used clinical pooled sensitivity and specificity data from systematic reviews of tests to indicate the likely performance of the tests analysed in this study is shown in Table 3.

Results

To examine the performance of the WHO catalogue of mutations and the mutations identified by molecular diagnostic tests compared to phenotypic DST, we constructed in silico versions of molecular diagnostic tests based on lists of mutations that they detect (Table 1). We examined all drugs for which a WHO endorsed molecular test is available, including first-line agents (rifampicin, isoniazid and pyrazinamide) fluoroquinolones (levofloxacin and moxifloxacin), injectable drugs (amikacin, capreomycin and kanamycin), and ethionamide.

Rifampicin. The WHO catalogue of mutations, which includes mutations outside of the rifampicin resistance-determining region (RRDR) of \( rpoB \), identified 93.8% of the phenotypically rifampicin-resistant isolates in the dataset (Fig. 1). This was higher than any of the other molecular tests (80.1–92.6%), which do not detect mutations outside of the \( rpoB \) RRDR.

Several molecular diagnostics can infer rifampicin resistance based upon detection of any mutation within the RRDR, but the tests differ in the exact genetic positions they target (Table 1). Despite these differences, there was no notable difference in sensitivity or specificity for the sets of mutations detected by Xpert MTB/RIF, Xpert MTB/RIF Ultra, Genoscholar NTM + MDRTB detection kit v2, GenoType MTB BDP plus ver 2.0, INNO LiPA MTB Rif, BD MAX MDRTB and Abbott Realtime MTB RIF/INH (Fig. 1). The in silico specificity of all molecular tests and the catalogue was high, at over 98%, and tests where specific mutations are detected were more specific than any resistance inferred test interpretation and the catalogue (Fig. 1). Notably, the Xpert MTB/RIF Ultra, which includes specific probes to exclude non-resistance conferring mutations in the RRDR to reduce

| Drug      | Total isolates | Phenotypic resistant isolates | Percentage phenotypic resistant isolates |
|-----------|----------------|------------------------------|----------------------------------------|
| Rifampicin| 34,416         | 9869                         | 28.7                                   |
| Isoniazid | 34,440         | 12,195                       | 35.4                                   |
| Pyrazinamide | 15,934      | 2337                         | 14.7                                   |
| Ethionamide | 14,009       | 2978                         | 21.3                                   |
| Levofloxacin | 18,386       | 3125                         | 17.0                                   |
| Moxifloxacin | 13,440       | 1880                         | 14.0                                   |
| Amikacin  | 17,093         | 1295                         | 7.6                                    |
| Capreomycin | 11,622        | 974                          | 8.4                                    |
| Kanamycin | 16,277         | 1488                         | 9.1                                    |

Table 2. Number of isolates with both WGS sequencing and phenotypic data for each drug. The total number of isolates does not sum to 38,111 because several isolates have phenotypic DST for multiple drugs.
false positive resistance predictions, did not significantly outperform GeneXpert MTB/RIF in terms of in silico specificity on this dataset. For tests where both resistance detected and resistance inferred interpretations are possible (Genoscholar NTM + MDRTB detection kit v2, GenoType MTBDRplus V2, INNO LiPA MTB Rif), the sensitivity was much lower at 80.1%, however the tests in question only detect four specific mutations. The in silico results showed that tests that identify a greater number of mutations to detect resistance performed better in terms of sensitivity (FluoroType MTBDR VER 2.0, cobas MTB RIF/INH and Truenat MTB/RIF achieved 90.9%, 89.2% and 87.8% sensitivity, respectively), though the mutations identified by these tests still had lower sensitivity than resistance-inferred interpretations.

**Isoniazid.** Similarly to rifampicin resistance, the catalogue mutations identified more isoniazid-resistant isolates in the dataset in the in silico analysis than any of the molecular diagnostic tests, with 91.2% sensitivity (Fig. 1). The specificity of the catalogue mutations was not significantly different from the molecular tests, except for resistance-detected and resistance inferred interpretations of RealTime MTB RIF/INH and for BD MAX TB.
MDRTB which had 0.37%, 0.35% and 0.35% higher specificity respectively. There was no significant difference in sensitivity between any of the in silico results for the molecular diagnostic tests, despite the differences in

![Figure 2.](image-url)

Sensitivity and specificity of WHO catalogue mutations and mutations detected by molecular diagnostic tests for predicting resistance to antitubercular drugs not used in first-line treatment. Error bars show 95% confidence intervals and point plots indicate performance of molecular diagnostic tests in clinical trials, these values are listed in Table 3. A full table of results is presented in Table S3. Drug acronyms: \( ETH \) ethionamide, \( LEV \) levofloxacin, \( MXF \) moxifloxacin, \( AMI \) amikacin, \( CAP \) capreomycin, \( KAN \) kanamycin.

MDRTB which had 0.37%, 0.35% and 0.35% higher specificity respectively. There was no significant difference in sensitivity between any of the in silico results for the molecular diagnostic tests, despite the differences in
mutations identified and genes probed, except for the resistance-detected based interpretation of RealTime MTB RIF/INH, which had lower sensitivity. Although there was no significant difference between in silico sensitivity compared to other molecular diagnostic tests, the Xpert MTB/XDR test identified the most phenotypically resistant isolates, with 89.4% sensitivity. However, it is notable that the in silico sensitivity of Xpert MTB/XDR for isoniazid resistance detection was lower than that seen clinically.

Pyrazinamide. For pyrazinamide, the mutations identified by Genoscholar PZA-TB II (which includes any mutation in pncA and up to 17 base pairs upstream) allowed a significantly higher proportion of pyrazinamide resistance to be identified in silico than the catalogue mutations, with 76.8% versus 72.2% sensitivity respectively (Fig. 1). However, the in silico sensitivity of Genoscholar PZA-TB II compared to phenotypic DST was much lower than the sensitivity seen in clinical evaluation. Despite inclusion of probes to identify commonly seen susceptible mutations to reduce false positive resistance calls, the specificity of the Genoscholar PZA-TB II was 2.0% lower in silico than that of the catalogue mutations.

Ethionamide. Molecular tests that identify inhA promoter mutations can diagnose resistance to ethionamide. For instance, the Xpert MTB/XDR test identified 47.3% of ethionamide resistance in silico (Fig. 2). The catalogue includes mutations in an additional gene target, ethA, and these additional mutations result in a significantly higher sensitivity of 75.6%. However, the catalogue mutations do have a significantly lower specificity than Xpert MTB/XDR, which targets inhA promoter mutations only for ethionamide resistance detection.

Table 3. Clinical performance of molecular diagnostic tests by drug. Where possible, sensitivities and specificities are pooled from multiple clinical studies in systematic reviews. Please see references for the methods used to determine the clinical sensitivity and specificity of tests.
Fluoroquinolones. For both moxifloxacin and levofloxacin, all the tests performed as well as the catalogue mutations in terms of in silico sensitivity (Fig. 2). The resistance inferred interpretation of GenoType MTBDRsl version 2 identified the highest proportion of phenotypic resistance, with 84.7% sensitivity for detecting levofloxacin and 88.2% sensitivity for detecting moxifloxacin resistance in silico, but this was not significantly different from the other molecular diagnostics. In terms of specificity, for moxifloxacin, there was no significant difference between the catalogue or any of the molecular diagnostic tests. For levofloxacin, there was a higher specificity in silico using the resistance detected interpretations of GenoType MTBDRsl v2 than the resistance inferred interpretation, and this was the only test with higher specificity than the catalogue. All the molecular diagnostic tests, for both levofloxacin and moxifloxacin resistance detection, had lower in silico sensitivity compared to the phenotypic DST than has been seen for the tests in clinical evaluation.

Aminoglycosides. The catalogue mutations did not have significantly higher sensitivity than any of the molecular diagnostic tests for detecting amikacin resistance (Fig. 2). The resistance inferred interpretation of Genotype MTBDRsl version 2 had the highest sensitivity of any test at 77.3%, but this was not significantly higher than any of the other tests. Specificity was high (\(> 99\%\)) for the catalogue mutations and all tests evaluated in silico. We found that both resistance detected and resistance inferred interpretations of GenoType MTBDRsl v1 were the only results that had higher specificity than the catalogue.

Although capreomycin and kanamycin are no longer recommended for treatment of rifampicin-resistant or multi-drug resistant tuberculosis, they may still be in use in settings where a better alternative are not yet available and therefore we have included them in our analyses. For capreomycin, the catalogue mutations, which include \(tlyA\) mutations, had significantly higher sensitivity in silico than all the molecular tests which only interrogate \(rrs\) and \(eis\) genes, and there was no significant difference in their specificities (Fig. 2). Of the molecular diagnostic tests, Genotype MTBDRsl version 2 identified the highest proportion of resistance, with 62.2% sensitivity in silico, however this was not significantly higher than any of the other molecular diagnostic tests.

For kanamycin, Xpert MTB/XDR and a resistance inferred interpretation of GenoType MTBDRsl version 2 had higher sensitivity than the other tests in silico, and no significant difference in sensitivity or specificity from the catalogue mutations (Fig. 2). The resistance inferred interpretation of GenoType MTBDRsl version 2 performed significantly better than the mutation only interpretation in terms of sensitivity, but for GenoType MTBDRsl version 1, where the \(eis\) promoter is not interrogated, both region and mutation interpretations performed equally. Specificity was \(> 98.0\%\) for all the tests and the catalogue mutations and the tests with the lowest sensitivity had higher specificities than the catalogue mutations.

The molecular diagnostic tests for resistance to aminoglycosides had lower in silico sensitivity than that seen clinically, bar Xpert MTB/XDR, which had similar sensitivities to the clinical measurements for detecting amikacin and capreomycin resistance (Fig. 2). The differences between in silico and clinical sensitivities may not be significant as the clinical data has large associated confidence intervals due to small sample sizes (Table 3).

Discussion

We found that for the drugs studied, the mutations detected by molecular diagnostic tests identify the majority of resistance, even in this diverse dataset, and performed comparably to the WHO mutation catalogue version 1.15 for moxifloxacin, levofloxacin, amikacin and kanamycin and better than the catalogue for pyrazinamide resistance detection (Figs. 1, 2). However, the sequencing approach had higher sensitivity in silico than any of the PCR-based diagnostics for rifampicin, isoniazid, ethionamide and capreomycin (Figs. 1, 2).

Molecular diagnostic tests perform similarly in silico compared to sequencing for identifying resistance to fluoroquinolones and two aminoglycosides. Specifically, for levofloxacin, moxifloxacin, amikacin and kanamycin, there was no significant difference between the in silico performance of the mutations covered by some molecular diagnostic tests and the catalogue (Fig. 2). This is an encouraging result, as areas of high TB burden may rely on molecular diagnostic tests as cheaper and more easily implementable alternatives to current sequencing approaches for certain drug resistance detection.

For pyrazinamide, the mutations identified by Genoscholar PZA II identified a significantly greater proportion of resistance than the catalogue (Fig. 1). Unlike with other drugs, there is no hotspot region of resistance mutations in \(pncA\), and thus there are many rare mutations conferring pyrazinamide resistance across the gene and promoter regions, which the Genoscholar PZA II probes fully cover (Table 1). The WHO mutations catalogue however, is a first iteration, and included only mutations with sufficiently high confidence scores that were included as ‘associated with resistance’ or ‘associated with resistance – interim’ and therefore rare resistance conferring mutations in \(pncA\) may not have been included.15,24 As more resistant samples are included in future iterations of the WHO mutations catalogue, it is likely that more rare mutations with ‘uncertain’ significance will be pushed over the threshold and be considered associated with resistance.

The difference in the in silico performance of molecular tests was most pronounced for molecular tests that covered greater numbers of mutations. Despite differences in the mutations covered by molecular diagnostic tests, for moxifloxacin, levofloxacin, amikacin and capreomycin there was no significant difference in in silico sensitivity between any of the tests (Fig. 2). However, we found that increasing the number of mutations probed by molecular diagnostic tests can improve sensitivity for detecting resistance for the other drugs, and this is best exemplified by the data for rifampicin (Fig. 1). When using four specific mutation probes to detect resistance, as for resistance detection interpretations of Genoscholar NTM + MDRB detection kit v2, GenoType MTBDRplus ver 2.0 and INNO LiPA Mtb Rif, 80.1% sensitivity was achieved. After increasing the number of specific mutations detected to 13, 18 and 32 as for Truenat MTB/RIF, cobas MTB/RIF, FluoroType
The in silico performance of molecular diagnostic tests for pyrazinamide, fluoroquinolone and aminoglycoside resistance is lower than their clinical performance. For pyrazinamide, amikacin, capreomycin, kanamycin, levofloxacin and moxifloxacin, in silico sensitivities of the molecular tests were generally lower than what has been reported in the literature (Figs. 1, 2). Resistance to these drugs is less common than for isoniazid and rifampicin, and therefore clinical resistant sample sizes are smaller and are likely to only contain the most prevalent resistance conferring mutations. However, the data used for our in silico analysis was drawn from a large sample set collected for the purpose of capturing rare phenotypes and diverse genetics for compiling the WHO mutations catalogue\textsuperscript{13,14}, and as such is likely to have greater diversity than we would expect to see in clinical trials, particularly if trial samples are drawn from homogenous geographical regions.

Sequencing has better in silico performance than molecular diagnostic tests for identifying phenotypic resistance to rifampicin, isoniazid, ethionamide and capreomycin and has additional benefits. Our analysis demonstrates that in silico sequencing-based DST performed better than in silico molecular diagnostic tests for rifampicin, isoniazid, ethionamide and capreomycin resistance detection (Figs. 1, 2). The advantage is that sequencing-based DST can be used to identify resistance across a broad genetic region allowing for resistance detection of drugs that have a more complicated genetic basis. For example, the Xpert MTB/XDR test, which detects inh\textsubscript{A} promoter mutations (important for resistance to both isoniazid and ethionamide\textsuperscript{6,28,29}) but none of the ethionamide resistance conferring mutations occurring elsewhere in the genome, only identified 47.3% of the phenotypic resistance present in the data. The catalogue, which includes mutations in the eth\textsubscript{A} gene in addition to inh\textsubscript{A}, fared significantly better at 75.6% sensitivity, and this difference highlights the importance of probing multiple gene targets when they are implicated in phenotypic resistance.

The higher sensitivity for rifampicin resistance detection of the WHO catalogue compared to PCR-based diagnostics in silico, may be due to the presence of resistance conferring mutations outside of the RRDR, such as rpoB I491F which are present in the catalogue but not detected by the molecular diagnostics (Table 1)\textsuperscript{9,15}. The rifampicin resistance associated rpoB I491F mutation is particularly prevalent in Southern Africa, meaning that molecular based diagnostic performance will vary based the prevalence of specific mutations in various geographical regions, whereas sequencing would include an exhaustive mutation set, reducing the impact of variations in regional prevalence.

There is a range of other benefits sequencing can provide in comparison to molecular diagnostic tests. Increased uptake and use of NGS, and especially WGS, will also allow new mutations to be identified as they emerge and will enable further associations with resistance to be made. As new resistance-associated mutations are added to the catalogue, molecular diagnostic tests will not be able to adapt their landscape for resistance detection easily or quickly, whereas sequencing would only need a bioinformatics update and, in the case of nGS platforms, the addition of a new primers if additional genes are needed. An additional benefit of sequencing is the ability to distinguish between mutations causing higher and lower levels of resistance as patients could benefit from different treatment regimens and dosages\textsuperscript{38}, this is straightforward with sequencing but is not possible with molecular tests that infer resistance. It is important to note that distinguishing between different levels of resistance is also possible for molecular diagnostic tests that use specific mutation probes\textsuperscript{39} and with the Xpert MTB/XDR test for isoniazid and fluoroquinolone resistance\textsuperscript{42}. Finally, while heteroresistance was not considered in this study, mixed populations can be detected and quantified using nGS\textsuperscript{56} and WGS\textsuperscript{57}, however we note that nGS may be the preferable option as the culturing step required for WGS could prevent the detection of mixed infections\textsuperscript{58}.

Comparison of in silico performance of mutations identified by the WHO 2021 catalogue and molecular diagnostic tests to phenotypic DST. Overall, mutations identified by the catalogue and available molecular diagnostic tests had higher sensitivity for detecting rifampicin resistance than other antitubercular drugs; several tests identified over 90% of rifampicin resistant isolates in the dataset. This illustrates how rifampicin resistance conferring mutations have been well characterised\textsuperscript{69–72} and rapid molecular tests are thus also well suited for identification of resistance.

For all drugs bar rifampicin and isoniazid, both molecular diagnostic test and catalogue sensitivity in silico were below 90%, the target product profile for the minimal criteria to identify resistance at peripheral centres\textsuperscript{48}. For pyrazinamide, ethionamide, amikacin and capreomycin, the in silico sensitivity of the catalogue and all molecular diagnostic tests particularly low, less than 80%. This illustrates the need for further work identifying mutations associated with resistance to these drugs. Overall, the sensitivity of the catalogue and all molecular diagnostic tests for fluoroquinolones is also low (Fig. 2), despite resistance being well attributed to key regions in the gyr\textsubscript{A} and gyr\textsubscript{B} genes\textsuperscript{7}. This could be driven by heterozygous calls at less than 90% fraction read support...
that were not identified in the isolates used in this analysis. Significant fluoroquinolone heteroresistance has been observed in \textit{M. tuberculosis} \textsuperscript{5,7}, yet clear thresholds for clinically significant heteroresistance have not been set. Our understanding of the genetic determinants of resistance is far from exhaustive, and complex genetic resistance signatures involving increased efflux, metabolic changes and membrane permeability have yet to be characterized, thus limiting the sensitivity of diagnostics.

The specificity of both the catalogue and the tests was generally high in silico, and in line with the WHO optimal requirements at over 98\% \textsuperscript{58}, for all drugs except for moxifloxacin, ethionamide and pyrazinamide (Figs. 1, 2). We attribute the low in silico specificity for moxifloxacin resistance detection to the use of historical data with critical concentrations that are no longer recommended for defining resistance\textsuperscript{64}.

Limitations. Because no other comparably large dataset was available the dataset used for the in silico analysis was the same dataset that used to create the catalogue and therefore the in silico performance of the catalogue may be an overestimate compared to real world performance, a limitation in our study. Also, to ensure a large sample set, the phenotypic data used was collected using different breakpoints. Continual updates to critical concentrations highlight reliability problems with the phenotypic DST standard\textsuperscript{51,64}. Furthermore, several isolates containing common and well characterised resistance conferring mutations, such as \textit{katG}S315T and \textit{rpoB}S450L, were reported as phenotypically susceptible to isoniazid and rifampicin. Possible explanations include labelling errors during phenotypic testing\textsuperscript{5,76}, or incorrect phenotypic DST. Despite the limitations associated with phenotypic DST, it will continue to be required as a first step to identify new resistance mechanisms and associations as these cannot be identified using sequencing alone.

Conclusion

In conclusion, NGS has many benefits in comparison to PCR-based molecular diagnostic tests. As NGS is not yet feasible as a universal solution to fit all DST needs, it is encouraging to find that the mutations probed by molecular PCR-based diagnostics capture a high proportion of drug resistance in this diverse dataset. We hope that this dataset, and the presented in silico method, could help quickly evaluate lists of mutations for new or revised drug resistance assays. Although there are many advantages and limitations to consider for any diagnostic test, it is clear that a variety of diagnostic tools, including both targeted molecular and NGS based solutions, and phenotypic DST to identify resistant isolates and associated resistance mechanisms, will be vital to improve detection of antibiotic resistance and ultimately eliminate TB.

Data availability

All data used and generated during this study are included in this published article (and its Supplementary Information files). Data analyzed in this study were a re-analysis of existing data, which are openly available at locations cited in the reference section, but the matched genetic and phenotypic data for all isolates, including European Nucleotide Archive accession numbers for all the raw sequencing data, are presented in Table S4.

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Mycobacterium tuberculosis is a common pathogen that causes tuberculosis (TB) and is the leading cause of death from a single infectious agent. Resistance to antituberculosis medications, particularly rifampicin and isoniazid, is a major concern in the management of TB. Various methods have been developed to detect drug resistance, including line probe assays (LPAs), molecular methods, and next-generation sequencing.

### Key Points

1. **Molecular Methods:**
   - **LPAs:** These assays are rapid and widely used due to their high sensitivity and specificity. However, they are limited to detecting mutations in specific genes.
   - **Whole-genome sequencing:** This method provides comprehensive information on drug resistance, including complex mutations and resistance gene copy number.

2. **Next-Generation Sequencing (NGS):**
   - **Whole-genome sequencing:** This approach is increasingly being used to detect drug resistance mutations.
   - **Targeted sequencing:** These methods target specific genes associated with drug resistance, such as *rpoB* and *pncA*

3. **Molecular Assays:**
   - **Truenat MTB-RIF Dx:** This assay is designed to detect rifampicin resistance directly from sputum.
   - **GenoType MTBDRsl:** This assay is used to detect resistance to second-line anti-tuberculosis drugs.

4. **Clinical Relevance:**
   - The accurate detection of drug resistance is crucial for effective treatment strategies and public health interventions.

5. **Future Directions:**
   - Integration of NGS with laboratory information systems to improve surveillance and control of TB.

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Author contributions
R.E.C. and A.S. conceived the study. A.B. performed the analysis and wrote the manuscript. R.E.C., M.S., S.B.G., T.M.W., S.U. and A.S. provided feedback and edits. R.E.C. supervised the work.

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Competing interests
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