Speeding up drug susceptibility testing in *Mycobacterium tuberculosis* using RNA biomarkers

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Summary

Background Efficient management of drug-resistant tuberculosis relies on fast diagnostics. To accelerate phenotypic drug susceptibility testing [pDST] for *Mycobacterium tuberculosis* [TB], we introduce TRACeR-TB, a test that infers drug resistance from antibiotic-specific mRNA biomarkers.

Methods To develop TRACeR-TB, target genes were first identified through RNA sequencing experiments conducted on two drug-exposed, susceptible strains for four antitubercular drugs. Based on these findings, we designed drug-specific multiplex Quantigene panels to quantify mRNA levels of 8–9 biomarkers per drug (class), directly from crude cell lysates. The performance of TRACeR-TB was compared to the widely used Mycobacteria Growth Indicator Tube [MGIT] pDST by subjecting 238 strains with diverse drug resistance profiles to both methods, and aligning results to genotypic data. Furthermore, we explored TRACeR-TB's potential for evaluating molecules that enhance antibiotic efficacy, and investigated its applicability in macrophage models to assess Mtb's intracellular stress responses to drugs.

Findings Antituberculosis drugs trigger distinct transcriptional stress responses in susceptible, but not resistant bacilli, enabling a differentiation of the antibiotic phenotype in only 6 h. Validation on 238 strains showed TRACeR-TB had 100% (95% CI: 93·1–100%) sensitivity and 89·5% (95% CI: 74·7–97·2%) specificity compared to, respectively, 82·3% (95% CI: 69·2%–91·5%) and 94·8% (95% CI: 81·9%–99·4%) for MGIT pDST. TRACeR-TB specificity is likely underestimated due to the inclusion of isolates harbouring uncharacterised mutations. TRACeR-TB demonstrated 100% concordance with MGIT for drugs with reliable MGIT outcomes (moxifloxacin and isoniazid). Additionally, its sensitivity outperformed current rifampicin testing, detecting resistance in all borderline-resistant strains that MGIT missed, and bedaquiline testing. Furthermore, the assay detected the predicted effect of a novel drug booster and the intracellular drug-induced stress in macrophage models, highlighting its potential for drug optimisation.

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Interpretation TRACeR-TB is a complementary addition to current DSTs and can have a substantial impact on the TB diagnostics field. This tool can also play a vital role in identifying resistance mutations, thereby closing gaps in genotypic knowledge, and contribute to drug discovery and development.

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Keywords: Tuberculosis; Antimicrobial resistance; Diagnosis; RNA; Biomarkers

Research in context

Evidence before this study

Current standard phenotypic drug susceptibility testing (pDST) of *Mycobacterium tuberculosis* takes up several weeks. While DNA-based methods have strongly expanded the TB diagnostic capacity worldwide, the primary identification of new resistance-causing mutations depends on pDST results. This will continue to be essential in the future as new therapeutic approaches are being developed and resistance mutations continue to emerge. Earlier work reported the detection of bacterial drug resistance through the use of RNA biomarkers. Given that transcriptomic changes are among the earliest cellular responses to drug exposure, we explored the transcriptional responses of MTB to drugs as a potential method to accelerate pDST. We conducted a PubMed search using terms like 'tuberculosis', 'Transcriptional Responses', 'gene expression', 'microarray', and 'RNA sequencing', identifying seven relevant studies on the transcriptional response of susceptible MTB under antibiotic pressure or external stress. Analysis of the studies' results indicates that similar stressors or drugs with comparable modes of action trigger analogous transcriptomic responses, highlighting genes that could serve as mRNA biomarkers for detecting MTB drug susceptibility. The project's initial searches, conducted in 2015 and 2016, and subsequent searches were restricted to articles published in English. Four more key studies on transcriptional changes in TB under antibiotic pressure were published after our initial literature search, and examined in our work. Additionally, the authors who conceptualised the detection of bacterial drug resistance using RNA biomarkers published a follow-up paper in 2019.

Added value of this study

Our work represents the development of a pDST for *M. tuberculosis* based on mRNA biomarkers. The results were compared to standard pDST, and genomic data provided further insights into the performance of predicting drug susceptibility. Some isolates with mutations known to confer resistance were missed by the standard pDST but were accurately classified as resistant using our rapid pDST. In addition to outperforming the sensitivity of the standard pDST, TRACeR-TB results give an indication on resistance levels, offering valuable additional information for strain/mutation phenotypic characterisation. Furthermore, some isolates were identified as resistant by both pDST methods but carried mutations with uncertain implications for resistance. TRACeR-TB provides additional insights into the phenotypic resistance of unknown mutations, improving the performance of drug susceptibility predictions based on genotypic data.

Implications of all the available evidence

For the purpose of advancing sequence-based technologies and next-generation molecular diagnostics, the WHO published a second version of its catalogue in 2023 including information on mutations associated with phenotypic drug resistance. Our approach could enhance the phenotypic characterisation of mutations and help bridge the gap between phenotype and genotype that urgently needs to be filled to improve and fasten therapy. TRACeR-TB differentiates between susceptible and resistant strains, including low- and high-level resistance, within six hours of drug exposure. Due to its scalability for high-throughput and short time span TRACeR-TB is also a promising tool for optimising antibiotic potentiators both *in vitro* and *in vivo*. We validated TRACeR-TB for several first- and second-line drugs, demonstrating the performance of using RNA biomarkers to investigate tuberculosis resistance. Therefore, rapid, reliable phenotypic DSTs like TRACeR-TB can have a major impact for TB diagnostics and but also for drug development.

Introduction

Despite decades of efforts and progress in the field, tuberculosis [TB] remains one of the world's most deadly infectious diseases.[1] Effective management of drug-resistant TB relies on rapid diagnosis of its antibiotic resistance profile. Drug resistance in *Mycobacterium tuberculosis* [MTB] emerges primarily through mutations in drug targets and/or activation mechanisms, enabling genetic prediction of drug resistance, and thereby paving the way for WHO-endorsed targeted amplification diagnostics like GeneXpert MTB/RIF and deep amplicon sequencing.[2,3] However, these assays inherently rely on knowledge of causative mutations, which remains vastly incomplete for repurposed and

new drugs like bedaquiline [BDQ].[4] Moreover, genetic tests cannot measure levels of resistance, nor detect non-heritable drug tolerance.[5] As a result, slow phenotypic growth inhibition tests remain the standard in the field, despite being suboptimal for BDQ and rifampicin [RMP] testing.[6,7] A fast phenotypic drug susceptibility test [pDST] would clearly not only have clinical benefit, it can make a key difference closing the genotypic/phenotypic knowledge gap and speed up *in vitro* lead optimisation of novel TB drugs.

Arguably one of the fastest bacterial phenotypic responses on external stimuli is the adaptation of a bacterium's transcriptional profile. Through a complex network of sigma factors, transcriptional repressors, small RNAs and riboswitches, the bacterial physiology is constantly reshaped to rapidly cope with external challenges.[8] The transcriptional response of MTB to antibiotic pressure has been widely studied, and shown to be drug(class)-specific and mainly steered by sigma factors and regulators belonging to the WhiB, GntR, WRE, Mar, and TetR families.[8–11] Seminal work showed that quantification of mRNA levels of stress-related genes in spiked cells normalised to untreated controls, can rapidly differentiate drug-susceptible from -resistant organisms.[12–14] Indeed, while an antibiotic elicits a drug-specific stress reaction in susceptible strains, expression levels of these particular genes stay unaltered in resistant strains.

Based on these principles and insights, we developed a pDST assay for TB, called TRACeR-TB (Fig. 1). We identified drug-specific mRNA biomarkers which are heavily induced or repressed by exposure to first- and second-line anti-TB drugs. Multiplex tests were designed to rapidly quantify mRNAs levels directly from MTB early culture lysates, and validated using both phenotypic and genotypic data. TRACeR-TB can distinguish susceptible from low- and high-level resistant strains after only six hours of drug exposure, and shows promising applications in the optimisation of novel antibiotic potentiators *in vitro* and *in vivo*.

Methods

TRACeR-TB test development and optimisation

Antibiotic-specific RNA biomarkers were identified through RNA sequencing of two pan-susceptible strains (H37Rv and ITM041195) and subsequent data analysis (Appendix S1 pp 1, 4, 18–20), followed by preliminary TRACeR-TB experiments to quantify biomarker expression in a set of 10–15 strains including drug-susceptible and -resistant isolates (Appendix S1 pp 1, 5). This process led to the selection of 5–6 responsive genes and 3 housekeeping genes (Appendix S1 pp 1, 21–23). TRACeR-TB experimental conditions were assessed during these preliminary experiments. These include the method for cell lysis and inactivation (Appendix S1 pp 1, 6, 24), starting culture and density (Appendix S1 pp 1, 7), drug incubation time (Appendix S1 p 8), and drugs concentrations (Appendix S1 pp 1, 18).

TRACeR-TB validation

Validation strategy and M. tuberculosis *isolates*

TRACeR-TB's diagnostic performance metrics (described in terms of sensitivity and specificity) were evaluated using genotypic outcomes and compared to the phenotypic DST outcomes of the widely used automated Mycobacteria Growth Indicator Tube [MGIT]® 960 system (Appendix S1 p 2). Genotypic data are the

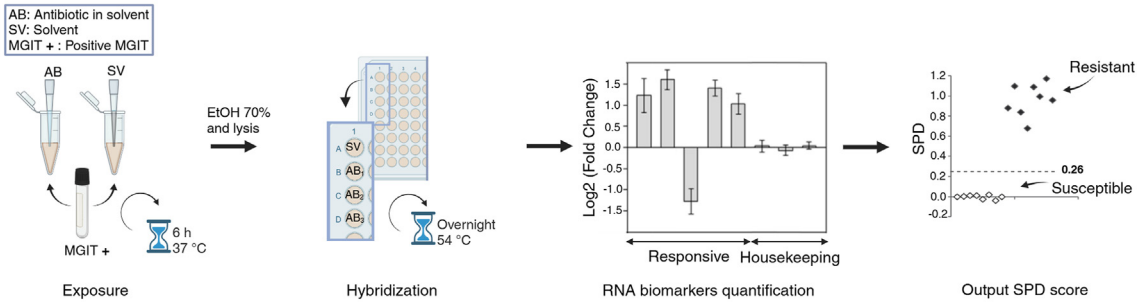

***Fig. 1:* Overview of the TRACeR-TB assay for rapid DST of *M. tuberculosis* based on RNA quantification.** To distinguish susceptible from resistant strains, we expose aliquots from culture positive Mycobacteria Growth Indicator Tube [MGIT] broths in exponential growth to an antibiotic or its solvent only for six hours at 37 °C. Following transcriptional arrest and a simple cell lysis step, we add a drug-specific oligonucleotide probe and magnetic bead set (QuantiGene technology, ThermoFisher Scientific) to the crude lysate, and the xMAP technology (Luminex Corporation) quantifies biomarker expression. A three-step normalization/transformation approach is used to convert raw fluorescent gene signals into fold-change values, employing housekeeping genes and solvent-exposed controls. We determine transcript induction or repression by comparing drug-exposed to solvent-exposed samples. Finally, the expression values are condensed into a single numeric value: the squared projected distance [SPD], calculated from a drug-specific core reference set.[14] Strains that are susceptible to a certain drug exhibit small SPDs close to 0, as a result of their distinctive, pronounced transcriptional responses, whereas antibiotic-resistant strains display higher SPDs near 1, indicative of little to no change in biomarkers expression. A threshold SPD at 0·26 allows the distinction between susceptible and resistant isolates. Created with Biorender.com.

outcome of an ISO 15089 accredited whole-genome sequencing analysis and/or commercially available genotypic rapid tests (GeneXpert, LPA).[15] We utilised existing collections of isolates accompanied by genomic and MGIT data (Appendix S1 p 1). For strains lacking this data, we performed WGS and/or MGIT DST (Appendix S1 p 2). Based on this genotypic and phenotypic information, we selected a diverse set of sensitive, mono-resistant, MDR, and XDR strains that encompass a broad mutational diversity. Details on the strains and the numbers of strains tested per drug are provided in Appendix S1 (p 25) and Appendix S2. Repeatability of the TRACeR-TB assay was evaluated by testing five times, starting each time from a new culture, a set of three or four isolates per drug including MGIT-susceptible and -resistant strains with different genotypic profiles (Appendix S1 p 9).

Antimicrobial agents

Moxifloxacin [MOX] hydrochloride (Sigma) and ofloxacin [OFX] (Sigma) were dissolved in 0·1 N NaOH; rifampicin [RMP] (Sigma), rifabutin [RFB] (Sigma), ethionamide [ETH] (Sigma), bedaquiline [BDQ] fumarate (Janssen Pharmaceutica NV) and SMARt751 in DMSO, and isoniazid [INH] (Sigma) in MilliQ water. Stock solutions were stored at –20 °C in small aliquots. Thawed solutions were kept at 2–8 °C for up to 3 days (RMP, RFB, BDQ) or 1 week (INH, OFX, MOX). Test concentrations and solvent-to-sample ratios (max. 1/500) are listed in Appendix S1 (p 18).

Antibiotic exposure experiments and inactivation

We inoculated fresh pre-cultures into MGIT (+0·8 ml Oleic acid-Albumin-Dextrose-Catalase [OADC]) to incubate in the BACTEC MGIT 960 system. Once growth units reached 1000 within 4 days of positivity (see Appendix S1 p 7), cultures were redistributed in 1 ml aliquots and exposed to antibiotics and their solvents for 6 h at 37 °C. For each strain/drug combination, we prepared three aliquots from the same culture-positive MGIT broth: lower drug concentration, higher drug concentration, and a solvent (negative) control. After centrifugation, 70% ethanol was added to preserve mRNA levels for differential gene expression analysis. See Appendix S1 (p 2) for greater detail. Pellets were stored at –80 °C for further processing.

Biomarker expression quantification

We employed the QuantiGene Plex 2.0 platform (ThermoFisher Scientific) to quantify differential gene expression in the antibiotic exposure experiments. Instead of amplifying mRNA, this technique amplifies the signal of oligonucleotide probes attached to magnetic beads (Luminex xMAP magnetic bead capture technology), providing a non-enzymatic alternative to qPCR. As mRNA levels are measured directly from crude lysates, the need for laborious mRNA extractions is obviated. We custom built drug-specific oligonucleotide probes and bead panels to quantify biomarker and housekeeping gene levels (Appendix S1 pp 21 and 22) and made them commercially available (Appendix S1 p 23). After drug exposure, cell pellets were lysed and the supernatant was processed using the QuantiGene Plex protocol, with background signal correction ensured by adding cell-free blanks. For more details, please refer to Appendix S1 (p 2).

Biomarker expression analysis and SPD threshold determination

We transformed the median fluorescent intensities (MFIs) obtained per biomarker or housekeeping gene from the Xponent Software (Luminex) to fold change (FC) values based on a three-step normalization/transformation procedure (Appendix S1 p 2). For each treatment/strain combination, we condensed the 5- to 6-biomarker log2 (FC) values (Appendix S3) into a single squared projected distance (SPD) using a custom-built drug-specific reference set of averaged transcriptional responses.[14] Strains susceptible to a tested drug have SPDs near 0 due to similar transcriptional responses, while resistant strains, showing little to no change compared to solvent controls, have SPDs near 1.

By applying machine learning based on the logistic regression algorithm, we were able to determine an SPD-threshold of 0·26 to distinguish susceptible from resistant strains (Appendix S1 p 2).

Ethics

No ethical approval was sought for the conduct of this work, because no primary data collection involving human or animal subjects took place. No metadata on any of the strains was used during this study, as the study focused solely on microbiological and genetic analyses.

Statistics

The sensitivity and specificity of TRACeR-TB and MGIT DST were calculated using a Clopper-Pearson 95% confidence interval (CI), based on the genotypic data of the isolates (Appendix S1 p 26). To determine the performance metrics of the assays, only strains harbouring mutations classified as group 1 (associated with resistance) or group 2 (associated with resistance—interim) in the WHO catalogue were considered truly resistant.[16] For more details, please refer to Appendix S1 (pp 3, 25) and Appendix S2. The sensitivity and specificity comparison between TRACeR-TB and MGIT DST was conducted using the Pearson Chi-Square test, with statistical significance set at $P < 0·05$ (Appendix S1 p 27).

The dataset utilised in this study is not homogeneous. Instead of targeting a specific number of strains per drug, we prioritised capturing a broad diversity of mutations, lineages, and strain backgrounds, with a primary focus on diversity.

Role of funders
Funders had no role in experiments design, data collection, data analyses, interpretation, or writing of report.

Results

Assay development

We designed TRACeR-TB to detect the effect of antibiotics targeting transcription (RMP and RFB), mycolic acid synthesis (INH), DNA replication (OFX and MOX), and ATP synthesis (BDQ). For each of these antibiotic classes, we identified specific stress-signatures (mRNA biomarkers) through a series of RNA sequencing experiments. As drugs with a similar mode of action are expected to provoke a similar transcriptomic response,[11,12,17] we restricted our analysis to a single antibiotic per class. These experiments identified between 210 and 817 induced genes and between 131 and 853 repressed genes per condition upon drug exposure (Appendix S1 pp 3, 19). Using criteria described in Appendix S1 (p 20), we selected for each drug-class five to six differentially expressed biomarker genes, along with three genes displaying unaffected mRNA levels during drug exposure (Fig. 2, Appendix S1 pp 2, 21 and 22).

In our test setup, these mRNA biomarkers are directly quantified from crude bacterial cell lysates, which allows to measure the bacterial stress response to antibiotics by comparing drug-exposed to solvent-exposed samples (Fig. 1). Through a series of optimisation experiments (Appendix S1 pp 7–9, 24), we achieved optimal results with six hours of drug exposure, transcriptional arrest by adding 70% Ethanol (vol/vol), and lysis with 0·5 mm silica/zirconia bead beating. Based on a machine learning algorithm using logistic regression and a training dataset for all drugs, we determined that a SPD threshold of 0·26 most effectively separated resistant from susceptible strains.[14] We developed a dedicated R shiny application to automate SPD calculation based on experimentally determined reference sets (Appendix S1 p 10; publicly available here: Transcriptional Response for Antimicrobial Resistance detection in TB | sciensano.be).

Assay validation

We evaluated two concentrations per drug: a lower dose for distinguishing resistant from susceptible strains (i.e., critical concentration [CC]) and a higher dose for assessing the level of resistance (Appendix S1 p 18). We compared the results to MGIT DST and determined both pDST performance metrics based on genotypic data (Appendix S1 pp 25–27).

Performance for fluoroquinolone-resistance detection

Fluoroquinolone resistance is acquired through mutations in the quinolone resistance-determining regions [QRDRs] of the gyrA gene and, less frequently, the *gyrB* gene.[18]

We validated TRACeR-TB against a panel of 97 MTB strains: 47 MGIT-susceptible to MOX and 50 MGIT-resistant to MOX harbouring common QRDR mutations, as well as some less common mutations in *gyrB* (Fig. 2, Appendix S1 pp 11, 25, and Appendix S2). The lower MOX concentration (CC_{MOX} = 0·0625 μg ml^{-1}) accurately identified all 50 MGIT-resistant strains with SPDs above the 0·26 threshold, and separated them from all MGIT-susceptible isolates. Among the 47 susceptible strains, we included a panel of clinical MTB strains representing eight lineages and sixteen sublineages to ensure that the measured stress response is independent of phylogenetic effects (Appendix S2).

The higher MOX dose (0·25 μg ml^{-1}) revealed mutation-specific levels of resistance. Most of the strains carrying GyrA mutations D94H/N/G or G88C maintained resistant/high SPDs to the high MOX dose, while those with GyrA_A90V or D94A, and GyrB_N499D or E501D mutations obtained low SPDs. These findings align with the WHO catalogue classification associating high- or low-level resistance to MOX with the respective mutations.[16] Interestingly, we identified a high level of resistance linked to a rare GyrB_R446H mutation, not comprised in public databases. Based on genotypic predictions, both pDSTs achieved a sensitivity and specificity of respectively 100% (95% confidence interval [CI]: 92·8–100%) and 97·9% (95% CI: 88·9–100%) (Appendix S1 p 26). In conclusion, MGIT DST and TRACeR-TB demonstrated comparable performance metrics and, in addition, they both identified a resistant phenotype in a strain lacking a characterised mutation (Fig. 2, Appendix S1 pp 11, 25 and Appendix S2).

Applying the same biomarkers to OFX testing, the TRACeR-TB accurately distinguished 50 MGIT-susceptible from 41 MGIT-resistant strains, exhibiting a separation highly concordant to the one obtained for MOX (Appendix S1 pp 12, 25 and Appendix S2). Notably, four isolates carrying GyrB_E501D exhibited a susceptible phenotype to OFX and a resistant phenotype to MOX. These results were confirmed phenotypically and supported by previous studies.[19,20]

Performance for rifamycin-resistance detection

Mutations in the RNA polymerase β subunit (*rpoB*) gene cause resistance to rifamycins and are mostly located in the rifampicin resistance determining region [RRDR].[21]

Our test panel covered 26 different *rpoB* resistance-associated variants [RAVs] (Appendix S2). At the critical concentration (0·25 μg ml^{-1}), the assay distinguished 77 strains bearing *rpoB* RAVs from 28 phylogenetically diverse RMP-susceptible strains (L1–7; L8 is RMP-resistant) bearing no RAVs. Intriguingly, TRACeR-TB flagged three lineage 1.1.2 MGIT-susceptible strains as resistant, presumably indicating yet unknown mRNA stability mechanisms in this sub-

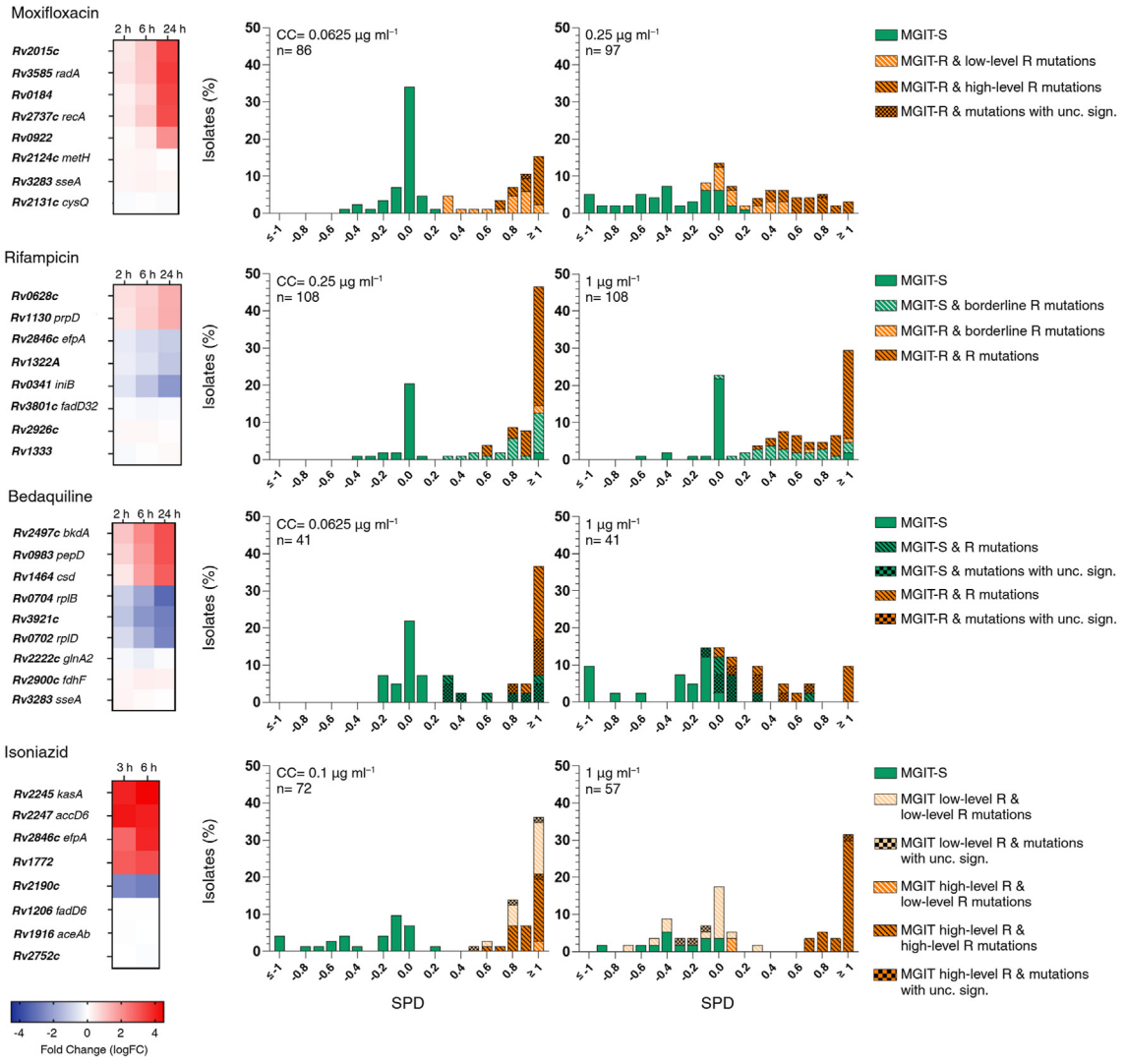

***Fig. 2:* TRACeR-TB biomarker selection and outcome in relation to MGIT and genotype.** Left panel. Transcriptional response of selected biomarkers of two pan-susceptible *M. tuberculosis* strains to four antitubercular drugs. The heatmap depicts their log2 FoldChange after 2, 3, 6, or 24 h of incubation with moxifloxacin (0·5 µg ml^{-1}), rifampicin (2 µg ml^{-1}), bedaquiline (3·2 µg ml^{-1}), and isoniazid (0·2 µg ml^{-1}). Right panels. The TRACeR-TB validation for moxifloxacin, rifampicin, bedaquiline, and isoniazid, with SPDs obtained after 6 h of exposure at the critical concentration [CC] (left side) and a higher concentration (right side) with the number of isolates tested [n]. MGIT-DST outcomes for the tested strains are presented in different colours: susceptible [MGIT-S] in green and resistant [MGIT-R] in orange. Isoniazid MGIT-DST was performed with two concentrations (0·1 and 0·4 µg ml^{-1}) to distinguish low/high-level of resistance [MGIT low/high-level R] in, respectively, light and dark orange in the figure. Genotypic outcomes for the tested strains are presented with different patterns. Drug resistance mutations are classified according to the latest WHO catalogue.[16] Mutations with a final confidence grading of "1) Assoc w R" and "2) Assoc w R – Interim" are labelled as (low/high) resistant [R]. Mutations with a final confidence grading of "3) Uncertain significance", or mutations not described in the catalogue are marked as "uncertain significance" [unc. sign.] and are only depicted as such if a resistant outcome was obtained in MGIT DST and/or TRACeR-TB. There is no separate labelling for mutations with a final confidence grading of "4) Not assoc w R - Interim" and "5) Not assoc w R". Borderline rifampicin resistance-conferring mutations are reported separately among the resistant strains (Appendix S2).[16]

lineage. As a result, the TRACeR-TB's specificity for the wild-type examined panel was 90·3% (95% CI: 74·3%–98%) compared to 100% (95% CI: 88·8–100%) for MGIT (Fig. 2, Appendix S1 pp 13, 25 and 26, 20 and Appendix S2).

It is known that less fit *rpoB* mutants with elevated, but not high, minimum inhibitory concentrations [MICs] grow too slowly in rapid liquid culture systems, potentially resulting in the underestimation of their resistance to RMP by MGIT.[7,22] TRACeR-TB

demonstrated a significantly superior sensitivity of 100% (95% CI: 95·3–100%) and detected these so-called 'borderline' RMP-resistant *rpoB* variants (H445L/N/S, D435Y, L452P, L430P, and I491F),[21] correctly identifying all of them as resistant, while MGIT missed 92·9% of them resulting in a sensitivity of 66·2% (95% CI: 54·6–76·6%) (Fig. 2, Appendix S1 pp 13, 25–27 and Appendix S2).

Next, we assessed resistance to RFB on a similar sample set. Apart from the three L1.1.2 strains, TRACeR-TB identified all 18 wild-type isolates as susceptible and all 64 strains carrying *rpoB* mutations as resistant (Appendix S1 pp 14, 25 and Appendix S2). Despite the lack of data confirming full cross-resistance with RMP, most high-SPD isolates carry mutations known to confer RFB resistance.[21,23,24]

Performance for bedaquiline-resistance detection

BDQ has become a core drug in MDR tuberculosis therapy, but susceptibility testing is challenging.[1] Apart from mutations in *atpE* associated with high-level resistance and *pepQ* (*rv2535c*), associated with low-level resistance,[25–28] BDQ resistance in clinical TB infections mostly stems from mutations in *mmpR5* (*rv0678*), a transcriptional repressor of the MmpS5-MmpL5 efflux pump.[29,30] Deciphering the genotype–phenotype correlation of *rv0678* mutations has been hampered by the diverse nature of *mmpR* mutations, many showing variable MICs.[30,31] Additionally, low-level resistance or MICs near the critical concentration contribute to inconsistent categorical phenotypic DST results.[31]

We evaluated the TRACeR-TB's performance using a panel of 17 wild-type strains covering various (sub-)lineages (L1–L8), 12 strains carrying 8 different mutations known as conferring BDQ resistance and 12 strains carrying 11 mutations not listed in the WHO catalogue or with "uncertain significance".[16] Fourteen strains were identified as resistant by both MGIT and TRACeR-TB, all presenting high SPD values (≥0·8) at the BDQ critical concentration (0·0625 μg ml^{-1}) and including only nine isolates carrying mutations known to confer BDQ-resistance. While MGIT flagged 27 strains as susceptible, TRACeR-TB identified only the 17 wild-type strains as susceptible (Fig. 2, Appendix S1 pp 15, 25 and Appendix S2). The majority of MGIT-susceptible but TRACeR-TB-resistant isolates exhibited a low SPD at the high BDQ dose (1 μg ml^{-1}), pointing to a low or intermediate level of resistance that MGIT failed to pick up. Additionally, MGIT-DST has given two different outcomes for the two strains carrying the AtpE_A28G (described as "associated with resistance")[16] but also for the two strains with the Rv0678_R135W mutation while they consistently obtained resistant SPDs. Therefore, TRACeR-TB sensitivity outperformed MGIT's sensitivity but might be less specific (sensitivity$_{TRACeR-TB}$ = 100%, 95% CI: 73·5%–100%, sensitivity$_{MGIT}$ = 75%, 95% CI: 42·8–94·5%, and specificity$_{TRACeR-TB}$ = 58·6%, 95% CI: 38·9–76·5% and specificity$_{MGIT}$ = 82·8%, 95% CI: 64·2–94·2%) although the discordance between phenotypic and genotypic outcomes may suggest the presence of strains harbouring resistance-conferring mutations that have not yet been characterised (Fig. 2, Appendix S1 pp 15, 25–27 and Appendix S2).

Performance for isoniazid-resistance detection

Resistance to INH involves about 15 genetic regions, with mutations in *fur*A-*kat*G, *fabG1-inhA*, and *ahp*C-*oxy*R accounting for the majority of resistance.[32]

Testing 72 strains at the INH critical concentration (0·1 μg ml^{-1}) correctly separated all 27 MGIT-susceptible strains from the 45 MGIT-resistant strains carrying mutations in *katG* and/or *inhA*, yielding similar sensitivity and specificity compared to MGIT-DST (Sensitivity = 100%, 95% CI: 91·4–100, and specificity = 87·1%, 95% CI: 70·2–96·4%). Similar to moxifloxacin, we observed that strains carrying mutations associated to high-level resistance displayed elevated SPDs for both INH concentrations, while most of the low-level INH-resistant strains showed low SPDs for the higher INH concentration (Fig. 2, Appendix S1 pp 16, 25 and 26 and Appendix S2). In addition, we observed resistant SPDs in four MGIT-resistant strains carrying *katG* mutations not listed in the WHO mutation catalogue,[16] underlining the ongoing challenge of accurately predicting antibiotic susceptibility from the genotype, even for a well-characterised drug such as INH.[33,34]

Repeatability of the TRACeR-TB assay

We evaluated the TRACeR-TB repeatability for MOX, RMP, and BDQ by testing five times MGIT-susceptible and -resistant strains with different genotypic profiles (Appendix S1 p 9). All wild-type MGIT-susceptible strains replicates yielded susceptible SPDs at both drug concentrations, while all MGIT-resistant replicates obtained resistant SPDs at the critical concentration, as did replicates of the two MGIT-susceptible/TRACeR-TB-resistant strains. In addition, the high MOX-concentration consistently identified all GyrA_A90V replicates with lower SPDs and the GyrA_D94G replicates with high SPDs. This data confirms excellent TRACeR-TB repeatability, including drug-specific observations.

Ability to detect heteroresistance in experimentally mixed populations

To evaluate the impact of heteroresistance or mixed infections on the robustness of the TRACeR-TB assay, we determined the limit of detection for identifying INH-resistant mutants in experimentally mixed populations. We mixed liquid cultures of MTB H37Rv and an INH-resistant KatG_S315T mutant at different ratios that were exposed for six hours to 1 μg ml^{-1} INH. The

magnitude of change in expression (log2 FC) gradually decreased with an increasing proportion of the resistant population (Appendix S1 p 17). SPDs indicated resistance once the mixed population contained approximately 70% of resistant bacilli.

Applications of TRACeR-TB in drug development

Lead molecule optimisation. To explore the potential of TRACeR-TB to speed up drug development and discovery, we conducted experiments using SMARt751, a member of the Small Molecules Aborting Resistance [SMARt] family of arylpiperidine. This small molecule has been shown to enhance the potency of ETH by activating an alternative enzymatic pathway that surpasses the standard bioactivation pathway of this pro-antibiotic.[35] We tested this compound in combination with ETH using the INH-specific TRACeR-TB probes and settings, based on the partially shared molecular mechanism between INH and ETH.[36] Alone, SMARt751 did not induce INH-inflicted stress, resulting in high (seemingly 'resistant') SPDs, in contrast to ETH (Fig. 3a). Combining ETH with SMARt751 further decreased these SPDs, indicating that this compound potentiates the activity of ETH in these ETH-susceptible strains. Most strikingly, ETH-resistant strains initially exhibited high (resistant) SPDs for both ETH concentrations, but clearly returned to a susceptible profile when combined with SMARt751.

Susceptibility testing in macrophages. In hit-to-lead optimisation, *in vivo* efficacy studies are pivotal. We tested whether the stress response measured by TRACeR-TB *in vitro* could be replicated *ex vivo*. Hereto we exposed uninfected THP-1 human macrophages (blank), MTB-infected macrophages and H37Rv MTB (positive control) to two doses of INH (0·1 μg ml^{-1} and 1 μg ml^{-1}) for six hours after 42 h of initial infection (Appendix S1 p 3). We optimised infection time, multiplicity of infection and cell disruption to ascertain enough material for expression-based stress detection, and we included several wash steps to eliminate extracellular MTB. We halted transcription after 48 h of infection (including six hours drug exposure), and quantified biomarker expression. Results showed expected up- and downregulation of biomarkers under INH exposure, indicating a similar stress response in intramacrophage compared to *in vitro* MTB (Fig. 3b). We observed no apparent difference in response when comparing the two INH doses. We obtained susceptible SPDs for all four conditions (low/high dose, *in vitro*/*ex vivo*), with a slightly less pronounced stress response in the *in vivo* setup compared to the *in vitro* setup. However, this did not affect global susceptibility outcomes for TRACeR-TB in either set-up.

Discussion

In this paper, we describe the design and validation of TRACeR-TB, which determines the drug-susceptibility profile of an MTB strain through multiplex quantification of transcriptional stress responses following six hours of drug exposure. With our dataset, we obtained a global sensitivity of 100% (95% CI: 93·1–100%) for TRACeR-TB and 82·3% (95% CI: 69·2%–91·5%) for

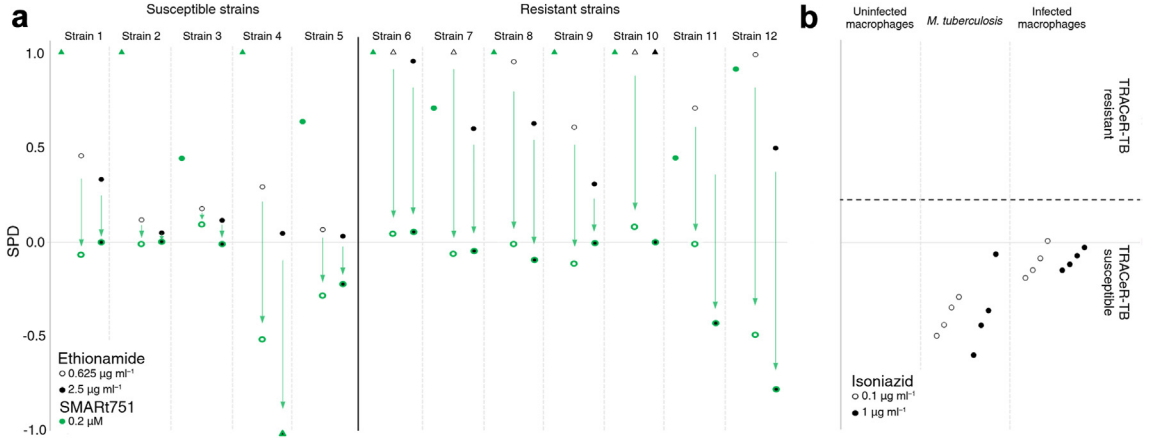

***Fig. 3:* Applications of TRACeR-TB in drug development.** (a) SMARt751 effect detection in TRACeR-TB.[37] SPDs obtained for five ethionamide MGIT-susceptible strains (strains 1–5) and seven ethionamide MGIT-resistant strains carrying the *inhA* c-15t mutation (strains 6 and 7) or an *ethA* mutation (strains 8–12). We tested each strain in five conditions: exposure to SMARt751, exposure to two ethionamide concentrations and exposure to two ethionamide concentrations combined with SMARt751 (green border). Triangles replace dots for values above 1 or below –1. Strain 1 is H37Rv, strains 2–7 are clinical strains from the Belgian National Reference Center for tuberculosis and Mycobacteria-Sciensano (2: S16BD02813, 3: S16BD05456, 4: S16BD05457, 5: S17BD00453, 6: 13MY0376- SRR13180422, and 7: 11MY0210- SRR13180328) and strains 8–12 are *in vitro* selected with *ethA* mutations provided by Sciensano (8: Y84C, 9: C253Stop, 10: G343A, 11: Y386Stop, and 12: W256Stop) (b) Susceptibility testing in macrophages. SPDs obtained for uninfected macrophages (no signal), H37Rv and macrophages infected by H37Rv exposed to 0·1 and 1 μg ml^{-1} of isoniazid.

MGIT-DST, while specificity stood at 89·5% (95% CI: 74·7–97·2%) compared to 94·8% (95% CI: 81·9%–99·4%) for MGIT-DST. This value is certainly an underestimation due to the inclusion of isolates harbouring uncharacterised mutations (Appendix S1 pp 25 and 26). Moreover, we demonstrated the test's ability to detect similar stress levels in macrophages, thereby confirming its capacity to also study intracellular drug responses. This insight opens the door to exploring the impact of host environmental changes, such as the detection of host-induced drug tolerance. For instance, Liu et al. observed that Mtb exhibited drug tolerance in activated macrophage models, while remaining susceptible in resting macrophages.[37]

In comparison with commonly used tests in the field, TRACeR-TB addresses two main issues. Firstly, WHO-endorsed phenotypic and rapid genotypic DSTs struggle with strains carrying non-RRDR mutations or borderline RMP-resistance conferring mutations, which have a global prevalence ranging geographically from 3·3 to 20·4%.[38–40] These strains are equally likely to be associated with poor treatment outcomes as the common *rpoB* mutations that cause high-level RMP resistance.[41] Given TRACeR-TB's independence of resistance mechanisms or cellular growth rates, it can outcompete classical testing in identifying strains with borderline *rpoB* mutations, such as those detected by WHO-recommended rapid molecular assays targeting only the RRDR region. Secondly, it also surpasses sensitivity of current BDQ-DST. Rapid and accurately detecting BDQ resistance is of major importance, given its crucial role in novel drug combinations for treating RMP-resistant TB. However, molecular mechanisms and mutations leading to BDQ resistance are not well-described and only provisional MIC breakpoints and interim critical concentrations exist for pDSTs. The absence of clear genotypic-phenotypic associations for BDQ resistance for the extensive range of mutations of "uncertain significance" complicates the implementation of genotypic susceptibility tests, still relying on phenotypic confirmation for such cases. A rapid and reliable RNA-based assay could enhance BDQ-pDST capacities, which is critical to prevent the further emergence and transmission of BDQ-resistant strains.

In general, the role of mutations in genes associated with phenotypic resistance to new and repurposed drugs like BDQ is poorly characterised, as MIC determination is not routine practice and mutations may occur infrequently. By analysing the effect of a low and high dose of a drug in TRACeR-TB, we demonstrated variable levels of resistance associated with distinct resistance mutations, which is particularly interesting for rare or unknown mutations that require proper characterisation. Moreover, the rapidity of our RNA-based assay can substantially accelerate research in identifying resistant isolates, thus aiding in bridging the genotypic/phenotypic knowledge gap.

A major advantage of the assay lies in its ability to easily evaluate the phenotype of hundreds of strains, far quicker than time consuming technologies such as the MGIT960 system. In our assay, drug-susceptibility profiles for a large panel of drugs can be obtained within 72 h once the culture reached the minimal growth units, as test results are read out in a high-throughput manner (96-well plates). By using two drug concentrations (standard set-up in our assay design), an indication of the level of resistance can be obtained. Furthermore, the brief drug exposure time of TRACeR-TB makes it particularly well-suited for less stable antibiotics (e.g., rifampicin and rifabutin), effectively addressing a limitation associated with traditional pDST.[42] In conjunction with whole genome sequencing [WGS], the rapidity of the assay can aid in characterising resistance mutations and be of great value to enrich or curate drug-resistance/WGS databases (e.g., WHO Catalogue of mutations).

This fast phenotypic assay can also be valuable in accelerating TB drug development. In this work, we analysed the predicted booster effect of SMARt751, which bypasses the classic activation pathway of ETH by initiating an alternative activation process. Not only can SMARt751 reverse ETH-acquired resistance, but also increase the basal sensitivity of MTB strains to ETH, a principle that could be documented by TRACeR-TB analysis, serving as proof-of-principle for utilising this assay to assess the potency of new drug boosters in development. Similarly, our data have shown that new compounds or formulations within the same class drug can be evaluated by employing the probes from the validated drug within that class, while for completely new drugs (or drug classes) probes can be selected based on RNA expression data.

One of the potential limitations of TRACeR-TB is its possibility to miss heteroresistance and/or mixed MTB subpopulations within a patient sample,[43,44] since the assay evaluates changes in gene expression as a reaction to drugs in susceptible strains. Due to this basic principle, the susceptible expression signature may mask the absence of change in expression by a resistant strain in a mixed population. Changes in biomarker expression caused by a stressed susceptible population are only overlaid when the resistant population reaches substantial proportions, rendering the RNA-based assay a relatively insensitive tool for the detection of resistant minority populations. It should be mentioned however, that determination of drug resistance profiles in mixed MTB populations remains a challenge for many DST methods. Moreover, to date, the assay has only been validated on cultured samples. Extending its application to direct testing on clinical sputum samples, would enhance its utility. However, achieving this requires the development, optimisation, and validation of a new pre-processing protocol.

In conclusion, we are convinced that the TRACeR-TB assay is a powerful addition to current DST assays and

can have a substantial impact on the TB diagnostics field. In a longer-term perspective, the translation of the assay to clinical practice will still require converting the current technology to a less laborious process and more cost-effective process. For example, conversion to RT-PCR for quantification of the biomarkers could reduce the number of washing steps currently needed and lower the cost (estimated in Appendix S1 p 28). In addition, training data for the machine learning model should be updated periodically, but as shown in our current work the potential is strong, which will hopefully also be interesting to potential partners for further clinical development.

In parallel, in a shorter-term perspective, we also envision applications in research settings, where this tool can play a vital role in identifying resistance mutations and help closing genotypic knowledge gaps, but may also serve in drug discovery and development.

Contributors

AVdB and PJC designed the project, secured funding and supervised the work. AVdB, RPB, and DTH performed the RNAseq experiments and analysis. AVdB performed the TRACeR-TB optimisation experiments with AS. AS and MM performed the TRACeR-TB experiments and analysis. AS, MM, and AVdB verified the underlying data. SD developed the machine learning model to determine the threshold and FDK the shiny app. FS, WF, and RB performed the macrophage infection model and analysis. PC supervised the work and provided helpful insights. VM, LR, and EMS provided the MTB isolates and AB the SMARt751. AVdB, PJC, MM, and AS prepared the manuscript with input from all authors. All authors read and approved the final version.

Data sharing statement

All relevant data are provided in this paper and the Supplementary Information. Source codes are available from the corresponding authors upon request.

Declaration of interests

Authors declare no conflict of interests regarding any financial and personal relationships with other people or organisations that could inappropriately influence our work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2025.105611.

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