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Agathe Jouet, Cyril Gaudin, Nelly Badalato, Caroline Allix-Béguet, Stéphanie Duthoy, et al.. Deep amplicon sequencing for culture-free prediction of susceptibility or resistance to 13 anti-tuberculous drugs. *European Respiratory Journal*, In press, 57, pp.2002338. 10.1183/13993003.02338-2020 . hal-03053938v1

HAL Id: hal-03053938

<https://hal.science/hal-03053938v1>

Submitted on 11 Dec 2020 (v1), last revised 20 Jul 2021 (v2)

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Early View

Original article

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Please cite this article as: Jouet A, Gaudin C, Badalato N, *et al.* Deep amplicon sequencing for culture-free prediction of susceptibility or resistance to 13 anti-tuberculous drugs. *Eur Respir J* 2020; in press (<https://doi.org/10.1183/13993003.02338-2020>).

This manuscript has recently been accepted for publication in the *European Respiratory Journal*. It is published here in its accepted form prior to copyediting and typesetting by our production team. After these production processes are complete and the authors have approved the resulting proofs, the article will move to the latest issue of the ERJ online.

Deep amplicon sequencing for culture-free prediction of susceptibility or resistance to 13 anti-tuberculous drugs

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Paper's message: The novel Deeplex Myc-TB molecular assay shows a high degree of accuracy for extensive prediction of susceptibility and resistance to 13 anti-tuberculous drugs, directly achievable without culture, which may enable fast, tailored tuberculosis treatment.

ABSTRACT

Conventional molecular tests for detecting *Mycobacterium tuberculosis* complex (MTBC) drug resistance on clinical samples cover a limited set of mutations. Whole genome sequencing (WGS) typically requires culture. Here, we evaluated the Deeplex Myc-TB targeted deep sequencing assay for prediction of resistance to 13 anti-tuberculous drugs/drug classes, directly applicable on sputum. With MTBC DNA tests, the limit of detection was 100-1000 genome copies for fixed resistance mutations. Deeplex Myc-TB captured in silico 97.1-99.3% of resistance phenotypes correctly predicted by WGS from 3651 MTBC genomes. On 429 isolates, the assay predicted 92.2% of 2369 first- and second-line phenotypes, with a sensitivity of 95.3% and specificity of 97.4%. Fifty-six of 69 (81.2%) residual discrepancies with phenotypic results involved pyrazinamide, ethambutol, and ethionamide, and low-level rifampicin- or isoniazid-resistance mutations, all notoriously prone to phenotypic testing variability. Only 2 of 91 (2.2%) resistance phenotypes undetected by Deeplex Myc-TB had known resistance-associated mutations by WGS analysis outside Deeplex Myc-TB targets. Phenotype predictions from Deeplex Myc-TB analysis directly on 109 sputa from a Djibouti survey matched those of MTBSeq/PhyResSE/Mykrobe, fed with WGS data from subsequent cultures, with a sensitivity of 93.5/98.5/93.1% and specificity of 98.5/97.2/95.3%. Most residual discordances involved gene deletions/indels and 3-12% heteroresistant calls undetected by WGS analysis, or natural pyrazinamide resistance of globally rare "*M. canettii*" strains then unreported by Deeplex Myc-TB. On 1494 arduous sputa from a Democratic Republic of the Congo survey, 14,902 of 19,422 (76.7%) possible susceptible or resistance phenotypes could be predicted culture-free. Deeplex Myc-TB may enable fast, tailored tuberculosis treatment.

INTRODUCTION

Important gaps remain for the diagnosis of drug-resistant tuberculosis (TB). Less than a third of the ~484,000 multidrug- (MDR) or rifampicin-resistant TB incident cases estimated in 2018 were diagnosed and treated [1]. Phenotypic drug-susceptibility testing (pDST) takes weeks of culture, while conventional molecular tests only identify common drug resistance mutations in few gene targets [2].

Whole genome sequencing (WGS) can effectively predict drug resistance or susceptibility of *Mycobacterium tuberculosis* complex (MTBC) strains [3–5]. However, its routine use for mycobacterial diagnosis typically requires primary culture [6]. Even if complex DNA enrichment procedures or thermoprotective DNA extraction are used, sequence coverage depths obtained by WGS directly on specimens often remain poor, even on samples with high bacterial loads [7,8]. This limits the sensitivity and/or the degree of confidence for detecting resistance mutations, especially when borne by minority populations (defining heteroresistance) that can be predictive of treatment failure [9].

Targeted, amplicon-based deep sequencing represents an attractive alternative [10]. Selective amplification of relevant gene regions prior to sequencing reduces both the required DNA amount and the interference of unrelated DNA sequences (human or from microbial flora). Sequence coverage depths and multiplexing of samples per sequencing run can be substantially increased [11].

A previously described amplicon-based assay targets pre-defined high confidence resistance variants in 6 *M. tuberculosis* genomic regions only [11,12]. In contrast, the targeted deep sequencing assay called Deeplex® Myc-TB (Genoscreen, Lille, France) targets full sequences (i.e. coding sequence plus part of promoter region) or (most) relevant regions of 18 MTBC drug resistance-associated genes, combined

with genomic targets for mycobacterial species identification and MTBC strain genotyping. Included in the assay, a fully automated web application is used for rapid and user-friendly analysis and interpretation of the sequencing data, obtained from Illumina sequencers. Variant detection is comprehensive, including not only mutations known to be associated with resistance or susceptibility, but also as yet uncharacterized mutations that can be confronted with drug-susceptibility phenotypes when available. This assay has already been used in national and regional TB drug resistance surveys, including those under the supervision of the World Health Organization (WHO) [13–15], prior to its extensive evaluation.

Here, we describe for the first time the details of the assay and evaluate its performances based on data from more than 4000 isolates and 1600 clinical specimens.

METHODS

Limit of detection

The limit of detection of Deeplex Myc-TB was evaluated using purified DNA from well-characterized MTBC strains of the WHO-TDR collection [16] (now included in the Belgian Culture Collection of Microorganisms (BCCM)). Serial dilutions were done after DNA quantification using the Qubit dsDNA HS assay (ThermoFisher, Waltham, Massachusetts, USA). A *M. intracellulare* strain from BCCM was used for evaluation of the limit of detection for NTM identification. After Deeplex Myc-TB amplification as per manual instructions, amplicon libraries were prepared using the Nextera XT kit and sequenced with 150bp paired-end reads using a MiSeq (Illumina, San Diego, California, USA). As for the subsequent parts, analyses were performed automatically using the integrated bioinformatics pipeline v1.3 implemented in the Deeplex Myc-TB web application (Supplementary Note S1).

In-silico analysis of drug resistance prediction versus WGS

The catalogue of resistance-associated variants included in Deeplex Myc-TB was compared to the resistance determinants and their associated resistance phenotypes found in both the training set of 2099 MTBC genomes and those retrieved in the validation set of 1552 MTBC genomes published in Walker *et al.* [3].

Deeplex Myc-TB phenotype prediction versus WGS and phenotypic testing

Comparisons of Deeplex Myc-TB variant detection and phenotype predictions versus WGS and pDST were performed using a set of 429 reference isolates, including 213 collected by the Belgian National TB Reference Center (Sciensano) between 2007 and 2015 (MDR strains) and between March and October 2013 (non MDR strains) and 216 from the WHO-TDR collection (BCCM) [16]. For the WHO-TDR collection,

pDST was performed by using the proportion method (on Löwenstein-Jensen or Middlebrook 7H11 agar medium, for first- or second-line drugs, respectively) with critical concentrations of 0.2, 40, 4, 2, 2, 6, 10 and 10 µg/ml isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, kanamycin, capreomycin and ethionamide, respectively, as described [16]. For the Sciensano strain set, pDST was routinely done using the BACTEC MGIT960 system and TB-eXiST application (Becton-Dickinson, Franklin Lakes, New Jersey, USA) for first-line drugs as per the manufacturer's instructions, and the radiometric BACTEC 460 TB (Becton-Dickinson) for second-line drugs including amikacin, ofloxacin, moxifloxacin, ethionamide and linezolid, as described in Pfyffer *et al.* [17] or Cambau *et al.* [18] for isolates before or after 2012, respectively. Deeplex Myc-TB testing was used as described above. WGS was performed at GenoScreen using the Nextera XT kit and 150bp paired-end reads on a HiSeq 2500 (Illumina) in Rapid Run mode. WGS analysis was performed using a Bowtie2-based pipeline with an initial threshold of 85% allele frequency for variant calling, with subsequent search for minority variants under low frequency detection mode, using at least one read in both forward and reverse direction, 5x read coverage and a minimal Phred score of 30 to call an allele with a minimal frequency of 3%. To avoid probable errors due to mislabeling of samples, Deeplex Myc-TB- and WGS-based phylogenetic lineage and spoligotype identifications were compared for consistency, and any isolate with more than three discordances between predicted and observed phenotypes was excluded from the analysis, as done elsewhere [3].

Deeplex Myc-TB on clinical specimens

Deeplex Myc-TB sequencing data from 109 sputum samples and WGS data from cultured isolates from the Djibouti survey were obtained as described in Tagliani *et*

a/ [13]. Phenotype prediction from WGS data was performed by MTBSeq v1.0.2 [19], PhyResSE v1.0 with resistance-associated variant database v29 [20] and Mykrobe v0.8.1 [21].

Deeplex Myc-TB sequencing data of 1494 sputum samples from the Democratic Republic of the Congo (DRC) survey were obtained as described in [22]. Briefly, after Ziehl-Neelsen staining and standard smear microscopy grading of acid-fast bacilli (AFB), sputa were stored and transported in 96% ethanol at a ratio of 1:1 at room temperature. DNA was extracted using a Maxwell 16 Low Elution Volume DNA Purification system (Madison, Wisconsin, USA), and analyzed with Deeplex Myc-TB kits using NextSeq and MiSeq platforms.

Data availability

Deeplex Myc-TB sequence reads were deposited in the Sequence Read Archive (SRA), National Center for Biotechnology Information (NCBI), under BioProject numbers PRJNA649788 (TDR/Sciensano), PRJNA633444 (Djibouti), PRJNA643157 and PRJNA643242 (Democratic Republic of Congo), PRJNA633380 (NTM) and PRJNA633106 (limit of detection analysis). WGS SRAs are available under BioProject numbers PRJEB31023 for the TDR collection, PRJNA393924 for the Djibouti dataset, and PRJEB25999 for the Sciensano collection. A detailed description of datasets used in this study is available in Supplementary Table 1.

RESULTS

Assay design, limit of detection, and mycobacterial species identification

The major gene targets associated with resistance to 13 first- and second-line anti-TB drugs/drug classes and the databases implemented in the web application are shown in Figure 1, Table 1 and Table 2. Further details on the assay design are provided in Supplementary Note S2, including the additional targets amplified in the single 24-plex PCR for mycobacterial species identification, spoligotyping/SNP typing and an internal control, and the analysis with the web application.

Table 1. Mycobacterial/MTBC genes or gene regions targeted by Deeplex Myc-TB. Positions of the reference sequences relative to the genome and genes of the *M. tuberculosis* H37Rv strain are indicated. Genome positions are indicated according to forward or reverse orientations of corresponding genes. For gene positions, – or + signs indicate positions in promoter or 3' regions relative to the +1 or last nucleotide of coding sequences, respectively. CDS, coding sequence; NA, not applicable for codons (positions outside a coding sequence, or in *rrs* or *rrl* rDNA regions).

Target	Genome positions	Gene positions	Codons
<i>rpoB1</i>	760957-761355	1151-1549	384-517
<i>rpoB2</i>	760280-760812	474-1006	158-336
<i>inhA</i>	1674287-1674880	86-679	29-227
<i>fabG1</i>	1673321-1673755	-119-316	NA-106
<i>katG</i>	2155858-2155140	254-972	85-324
<i>ahpC</i>	2726030-2726585	-163-393	NA-131
<i>pncA</i>	2289301-2288672	-60+9	Full CDS
<i>embB</i>	4247376-4248065	863-1552	288-518
<i>gidB</i>	4408185-4407411	18+117	6-NA
<i>rpsL</i>	781536-781979	-24+45	Full CDS
<i>rrs1</i>	1472561-1473417	716-1572	NA
<i>rrs2</i>	1471848-1472524	3-679	NA
<i>eis</i>	2715528-2715171	-196-162	NA-54
<i>tlyA</i>	1917811-1918750	-129+4	Full CDS
<i>gyrA</i>	7377-7754	76-453	26-151
<i>gyrB</i>	6298-6943	1059-1704	353-568
<i>ethA</i>	4327482-4325951	-9+53	Full CDS
<i>rplC</i>	801108-801483	300+21	100-NA
<i>rrl</i>	1475923-1476625	2266-2968	NA
<i>Rv0678</i>	778976-779539	-14+52	Full CDS
<i>hsp65</i>	528772-529172	165-565	55-189

Table 2. Databases implemented in the Deeplex Myc-TB web application for mycobacterial species identification, MTBC genotyping and drug susceptibility and drug resistance prediction. For the latter, priority is given to the collaborative, curated database ReSeqTB when detected variants are known to this database.

Database use	Database name	Reference
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Species identification	<i>hsp65</i>	[23]
Spoligotyping	SITVITWEB	[24]
Lineage identification	Coll	[25]
	PhyResSE	[20]
	Walker	[3]
Drug resistance prediction	Miotto	[26]
	PhyResSE	[20]
	Walker	[3]
	ReSeqTB	[27]

As Deeplex Myc-TB does not depend on a specific DNA extraction method, the assay's limit of detection was estimated as the fraction of detectable sequence variants in 4 replicated analyses using serially diluted purified, pre-quantified genomic DNA from three well-characterized MTBC strains, and a mixture of two strains at a 5-95% ratio. All (near-)fixed resistance variants were detectable with 10^4 and 10^3 genomes, and 83.3% with 10^2 genomes (complete variant profiles obtained for 13/16 tests, Figure 2). For resistance variant at 5% frequency, these fractions were 100% with 10^4 genomes, and 81.3% (with complete minor variant profiles in 3 out of 4 tests) and 43.8% (none with a complete minor variant profile) with 10^3 and 10^2 genomes, respectively. Fixed and minority variants were not detected with 10^1 genomes only (for limit of detection for MTBC and NTM identification, see Supplementary Figure S1-S2 and Supplementary Note S1).

Out of 370 strains from 73 different NTM species/species complexes tested using DNA extracted from culture, 292 strains were identified at (sub)species or species complex levels by both reference and Deeplex Myc-TB testing. Of these, 274 (93.5%) were correctly identified at (sub)species or species complex level by Deeplex Myc-TB (Figure S3, Supplementary Table S2 and Supplementary Note S3 for methods and details). The 18 (6.2%) strains that had taxonomically discordant results even at complex level between both methods mostly consist of single discordant cases among otherwise partially/fully concordant strains for a species

(e.g. *M. ulcerans*, n=1/13; *M. kansasii*, n=1/14), or species rarely involved in infections (e.g. *M. peregrinum*; n=2). However, for some of these few individual isolates of otherwise well identified species, the correctness of the Deeplex Myc-TB identification was actually often possible or probable, as conflicting or ambiguous identifications were seen between the *rpoB* and 16S rDNA reference probes, with one or the other partially or fully matching the Deeplex Myc-TB result (e.g. for *M. kansasii* vs *M. gastri*, Supplementary Table S2). The same held frequently true for the 16 residual isolates with discrepant subspecies within a matching complex (e.g. *M. intracellulare* vs *M. chimaera*).

Resistance variant detection versus WGS in silico

We evaluated, *in silico*, the ability of Deeplex Myc-TB to capture 120 anti-tuberculous drug resistance-determining mutations spread across 14 genes, along with their concurrent first- and second-line resistance phenotypes, algorithmically identified in a WGS study by Walker *et al.* [3]. Of these 120 resistance determinants, 106 (88.3%) are included in the Deeplex Myc-TB targets and variant catalogue (Supplementary Table S3), spread across 13 of the above 14 genes, the exception being *rpsA*, a minor target associated with pyrazinamide resistance. With these 106 variants, 644 of 663 (97.1%) resistant phenotypes predicted by WGS in the Walker's training set of 2099 MTBC isolates were retrieved. Likewise, 53 of 58 resistance-determining mutations from the training set that were retrieved in the Walker's validation set of 1552 isolates were captured by Deeplex Myc-TB, enabling the prediction of 1199 out of 1207 (99.3%) concurrent resistant phenotypes in this dataset (Supplementary Table S4).

Phenotype prediction on isolates versus WGS and phenotypic testing

We compared the ability of both Deeplex Myc-TB and Illumina-based WGS analysis to detect variants in DNA extracts from 429 MTBC strains. Of the 2403 variants identified in the Deeplex Myc-TB targets by either method, 2373 (98.8%; 2293 SNPs and all 80 indels), including 797 (99.9%) resistance variants, were detected by both Deeplex and our WGS pipeline under low frequency mode (validated for accurate SNP calling in accordance with recent guidelines [28]). The remaining 30 (1.2%) SNPs were all minority variants mostly with frequencies of ~3-10% only identified by targeted deep sequencing, including one mutation associated with resistance (to ethambutol; Supplementary Note S4; Supplementary Table S5).

Deeplex Myc-TB drug-susceptibility predictions based on these 2403 variants were compared to pDST results. In this set, 268 isolates were phenotypically resistant to at least one drug including 156 MDR and 6 extensively drug resistant isolates, resulting in 664 resistant and 1705 susceptible phenotypes. Of these 2369 phenotypes, 2184 (92.1%) were predicted by Deeplex Myc-TB with a mean sensitivity of 95.6% and a mean specificity of 97.4% (Table 3, Supplementary Table S6). The remaining 185 phenotypes (7.9%) could not be predicted due to the presence of mutations uncharacterized in the variant database. When results were stratified by type of phenotypic method used as a reference, the concordance with genotypic predictions was slightly higher for phenotypes tested by liquid culture (Supplementary Table S7) compared to those tested by solid culture (Supplementary Table S8), for the three drugs principally assayed with both types of methods (rifampicin, isoniazid, ethambutol).

Table 3. Deeplex Myc-TB phenotype predictions versus pDST on 429 isolates from the WHO-TDR and the Sciensano-Belgian National TB Reference Center collections. Deeplex Myc-TB predictions were compared to phenotypes separately for each drug,

across isolates with this data available. The unit of analysis was therefore a phenotype, not an isolate.

	Phenotypically resistant				Total	Phenotypically susceptible				Total	All		Excl. uncharacterized		Uncharacterized
	Genotype					Genotype					Sensitivity	Specificity	Sensitivity	Specificity	
	R	R _h	S	U		R	R _h	S	U						
Rifampicin	159	0	1	2	162	3	0	253	6	262	98.1 (94.7, 99.4)	98.9 (96.7, 98.2)	99.4 (96.5, 99.9)	98.8 (96.6, 99.6)	1.9%
Isoniazid*	176	0	3	8	187	3	0	200	34	237	94.1 (89.8, 96.7)	98.7 (96.3, 99.6)	98.3 (95.2, 99.4)	98.5 (95.7, 99.5)	9.9%
Pyrazinamide [#]	39	3	7	4	53	0	0	146	5	151	79.2 (66.5, 88.0)	100 (97.5, 100)	85.7 (73.3, 92.9)	100 (97.4, 100)	4.4%
Ethambutol	95	0	8	5	108	26	2	285	3	316	88 (80.5, 92.8)	91.1 (87.5, 93.8)	92.2 (85.4, 96.0)	91.1 (87.4, 93.7)	1.9%
Streptomycin	49	0	5	36	90	1	0	91	33	125	54.4 (44.2, 64.3)	99.2 (95.6, 99.9)	90.7 (80.1, 96.0)	98.9 (94.1, 99.8)	32.1%
Fluoroquinolones	17	1	1	2	21	0	0	183	13	196	85.7 (65.4, 95.0)	100 (98.1, 100)	94.7 (75.4, 99.1)	100 (97.9, 100)	6.9%
Amikacin	9	0	0	0	9	0	0	184	14	198	100 (70.1, 100)	100 (98.1, 100)	100 (70.1, 100)	100 (97.9, 100)	6.8%
Kanamycin	2	1	1	0	4	0	0	3	0	3	75 (30.1, 95.4)	100 (43.9, 100)	75 (30.1, 95.4)	100 (15.0, 85.0)	0
Capreomycin	2	1	1	0	4	0	0	5	0	5	75 (30.1, 95.4)	100 (56.6, 100)	75 (30.1, 95.4)	100 (15.0, 85.0)	0
Ethionamide	18	1	1	6	26	6	0	172	12	190	73.1 (53.9, 86.3)	96.8 (93.3, 98.5)	95 (76.4, 99.1)	98.6 (92.8, 98.4)	8.3%
Linezolid	0	0	0	0	0	0	0	20	2	22	NA	100 (85.1, 100)	NA	100 (83.8, 100)	9.1%
Total	566	7	28	63	664	39	2	1542	122	1705	86.3 (83.5, 88.7)	97.6 (96.7, 98.2)	95.3 (93.3, 96.8)	97.4 (96.5, 98.1)	7.8%

R: detection of at least one fixed resistance-associated mutation; R_h: detection of resistance due to a minority variant (heteroresistance); S: detection of mutations known not to be associated with resistance (phylogenetic, benign, synonymous) or no mutation detected; U: detection of at least one non-synonymous uncharacterized mutation in the absence of a resistance-associated mutation. Fluoroquinolones include ofloxacin and moxifloxacin. Results for bedaquiline/clofazimine are not shown, as there was no pDST comparator.

*A beta version of the Deeplex Myc-TB kit used for this analysis did not yet include the *ahpC* promoter region including two isoniazid resistance-associated mutations G-48A and C-57T (Supplementary Note S2). We checked for these two mutations in the WGS dataset and found only two samples (TB-TDR0013 and TB-TDR-0160) with a fixed G-48A mutation. They were considered in this comparison to reflect results as obtained with the up-to-date version containing this region, as Deeplex Myc-TB captured 100% of the variants detected by WGS in the initial *ahpC* gene part and all other gene targets in this dataset (see text).

#A simulation of the inclusion of additional pyrazinamide resistance-associated mutations published in Yadon *et al.* [29] (not incorporated in the ReSeqTB and Deeplex Myc-TB databases) resulted in identification of only three additional resistance mutations, classified as uncharacterised by Deeplex Myc-TB. However, out of these three mutations, two were found in pyrazinamide susceptible phenotypes and only one in a pyrazinamide resistant phenotype.

The proportion of resistant phenotypes accurately predicted as resistant by Deeplex Myc-TB was above 90% (90.7% for streptomycin-100% for amikacin) for most individual drugs, except for pyrazinamide (85.7%), kanamycin and capreomycin (75%, but reflecting 4 isolates only in both cases) (Table 3). For the MDR-defining compounds rifampicin and isoniazid, 159/160 (99.4%) and 176/179 (98.3%) isolates with resistance variants were correctly classified resistant, accounting for 98.1% and 94.1% of all rifampicin- and isoniazid-resistant isolates, respectively. For pyrazinamide, although all (42/42, 100%) isolates containing resistance variants for this drug were correctly predicted as resistant, these accounted for only 79.2% of all

isolates with phenotypic resistance to pyrazinamide. Of note, 7 minority resistance variants (with frequencies of 21.1 to 77%), successfully predicted resistance to pyrazinamide (n=3), fluoroquinolones, kanamycin, capreomycin and ethionamide (n=1 each).

Of the 601 resistant phenotypes with Deeplex Myc-TB predictions (i.e. not uncharacterized), only 28 (4.7%) were predicted susceptible due the absence of resistance-associated mutation in the Deeplex targets. In these phenotypes, the possible presence of high confidence resistance-associated mutations was searched in 13 secondary resistance-associated gene targets, outside the Deeplex Myc-TB target regions (e.g. the *embA* promoter region including e.g. the C-12T, C-16T mutations, or the extended *ethA* promoter region including e.g. the T-11C mutation; Supplementary Table S9). WGS analysis detected a high confidence resistance mutation in these regions for only one of these phenotypes (ethionamide resistant; L203L in *fabG1* [4]; Supplementary Table S9). Likewise, *fabG1* L203L was the sole established resistance-associated mutation (for an isoniazid resistant phenotype) detected by WGS outside of the assay's targets in 63 phenotypes uncharacterized by Deeplex Myc-TB.

Of the 1583 susceptible phenotypes with prediction, only 41 (2.6%) were discordantly predicted as resistant. They all involved ethambutol and *embB* mutations, ethionamide and *ethA* frameshift-causing indels (mechanistically expected to cause ethionamide resistance [30]), or known low-level isoniazid (*inhA* S94A, *ahpC* G-48A), rifampicin (*rpoB* L452P, H445N and D435Y) or streptomycin (*gidB* A138V) resistance mutations, all notoriously associated with poor phenotypic reproducibility [3,27,31–33] (Supplementary Note S4).

As acquisition of resistance to isoniazid is generally the first step towards drug resistance [34], a predicted susceptibility to isoniazid could *a contrario* be considered to predict susceptibility to other first-line drugs, for which gene targets have no drug resistance mutations but contain uncharacterized mutations, as shown for WGS-based analysis [4]. When doing so for Deeplex Myc-TB predictions, the diagnostic performance could be further improved, with proportions of uncharacterized phenotypes reduced to 0.5-2.5% for rifampicin, ethambutol and pyrazinamide, at cost of a single incorrect prediction of a susceptible phenotype for each of these drugs (Supplementary Note S5; Supplementary Table S10). This conditional interpretation is left to the user's decision, and not implemented in the Deeplex Myc-TB analysis algorithm.

Deeplex Myc-TB on clinical specimens

We compared variant detection and phenotype predictions based on available Deeplex Myc-TB sequencing data directly obtained from 109 clinical specimens from a nationwide survey conducted in Djibouti, versus analysis of WGS data obtained after culturing [13]. Overall, 693 of 752 total variants (92.2%) were detected by both Deeplex Myc-TB and our WGS pipeline used under low frequency detection mode. Of 59 (7.8%) discordances, all but one (a fixed *pncA* SNP not detected by Deeplex, albeit on a well-covered position) were minority variants undetected by this WGS pipeline, consistent with the average coverage depth of 3,921x at Deeplex targets by Deeplex Myc-TB versus 57x by WGS (Supplementary Note S6, Supplementary Table S11).

Deeplex Myc-TB phenotype predictions were matched to those obtained with WGS data independently analyzed with the WGS-based analysis tools PhyResSE [20], Mykrobe [21] and MTBSeq [19]. In contrast to PhyResSE and Mykrobe, MTBSeq

requires bioinformatic skills for local implementation and use. However, it has a more complete resistance mutation panel [19] and was therefore used as a primary reference in the comparison.

With the 1150 predicted phenotypes by Deeplex Myc-TB (155 resistant, 995 susceptible), the mean sensitivity and specificity versus available MTBSeq predictions were 93.5% and 98.5%, respectively (Table 4). One hundred and eight additional phenotypes (8.6%) were not predicted by Deeplex Myc-TB due to the presence of uncharacterized mutations. Agreement on resistance prediction was 100% for all applicable drugs, except for rifampicin (93.5%) and pyrazinamide (71.4%). The rifampicin score resulted from probable differences between primary samples directly tested by Deeplex Myc-TB and cultures used for WGS analysis. Indeed, two resistance predictions by MTBSeq (also by PhyResSE and Mykrobe; Figure 3) reflected detection of two minor resistance-associated variants in *rpoB* that were undetected by Deeplex Myc-TB despite high coverage depths at these positions, suggesting genotypic heterogeneity/contamination introduced/amplified after sputum processing, during subsequent culturing or WGS (Supplementary Note S7). For pyrazinamide, the lower sensitivity of Deeplex Myc-TB versus MTBSeq mostly resulted from different interpretation of some sequence variants or different regions interrogated in "*M. canettii*" isolates, which are naturally resistant to pyrazinamide and locally prevalent in and highly restricted to Djibouti [35]. Deeplex Myc-TB did identify 7 *M. canettii*-containing samples based on the phylogenetic SNP *pncA* A46A, but did not (then) explicitly predict resistance to pyrazinamide in contrast to MTBSeq based on *panD* M117T (and Mykrobe, based on *pncA* A46A; Figure 3 and Supplementary Note S8).

Conversely, 15 of the 995 (1.5%) phenotypes predicted as susceptible by MTBSeq (excluding uncharacterized phenotypes by Deeplex Myc-TB) were identified as resistant by Deeplex Myc-TB (Table 4). Of these, 10 (66.6%) discordances were due to minority resistance-associated variants at 3.3-12.8% only detected in sputa by Deeplex Myc-TB (Figure 3; Supplementary Note S7). Importantly, 8 out of these 10 minority variants co-occurred with one or more minority resistance and/or phylogenetic variants within an individual sputum. This further supports true-positive variant calls reflecting genuine mixed strains and/or combined heteroresistance detected by targeted deep sequencing, missed by WGS analysis due to lower coverage depth or potential culture bias. The five remaining discordant resistance predictions resulted from divergent variant interpretation, involving one *ethA* frameshift-causing indel (mechanistically expected to cause ethionamide resistance, see above), two debated *embB* mutations and a *gidB* mutation (n=2) associated with low-level streptomycin resistance [18] (Supplementary Note S7), which were associated with resistance by both Deeplex Myc-TB and PhyResSE and/or Mykrobe, but not by MTBSeq (Figure 3).

Mean sensitivity and specificity of the phenotypes predicted by Deeplex Myc-TB versus available PhyResSE and Mykrobe predictions was 98.5/93.1% and 97.2/95.3%, respectively (see Supplementary Note S8; Supplementary Tables S12 and S13).

Table 4. Phenotype predictions by direct Deeplex Myc-TB analysis of DNA from 109 clinical specimens versus phenotype predictions by MTBSeq with WGS data from culture. Deeplex Myc-TB sequencing and WGS data originate from a report of a national TB drug resistance survey conducted in Djibouti.

MTBSeq resistant	MTBSeq susceptible	All	Excl. uncharacterized	Uncharacterized
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	Genotype				Total	Genotype				Total	Sensitivity	Specificity	Sensitivity	Specificity	
	R	R _h	S	U		R	R _h	S	U						
Rifampicin	29	0	2	1	32	0	0	75	2	77	90.6 (75.8, 96.8)	100 (95.2, 100)	93.5 (79.3, 98.2)	100 (95.1, 100)	2.8%
Isoniazid	31	0	0	0	31	0	2	71	5	78	100 (89.0, 100)	97.4 (91.1, 99.3)	100 (89.0, 100)	97.3 (90.5, 99.2)	4.6%
Pyrazinamide	18	2	8	3	31	0	3	73	1	77	64.5 (46.9, 78.9)	96.1 (89.2, 98.7)	71.4 (52.9, 84.7)	96.1 (89.0, 98.6)	3.7%
Ethambutol	21	1	0	1	23	2	2	79	3	86	95.7 (79.0, 99.2)	95.3 (88.6, 98.2)	100 (85.1, 100)	95.2 (88.3, 98.1)	3.7%
Streptomycin	14	0	0	0	14	2	2	35	23	62	100 (78.5, 100)	93.5 (84.6, 97.5)	100 (78.5, 100)	89.7 (76.4, 95.9)	30.3%
Fluoroquinolones	0	0	0	0	0	0	1	104	4	109	NA	99.1 (95.0, 99.8)	NA	99 (94.8, 99.8)	3.7%
Amikacin	5	0	0	0	5	0	0	67	1	68	100 (56.6, 100)	100 (94.6, 100)	100 (56.6, 100)	100 (94.6, 100)	1.4%
Kanamycin	5	0	0	0	5	0	0	66	2	68	100 (56.6, 100)	100 (94.6, 100)	100 (56.6, 100)	100 (94.5, 100)	2.7%
Capreomycin	12	0	0	0	12	0	0	59	4	63	100 (75.8, 100)	100 (94.2, 100)	100 (75.8, 100)	100 (93.9, 100)	5.3%
Ethionamide	7	0	0	4	11	1	0	66	31	98	63.6 (35.4, 84.8)	99 (94.4, 98.2)	100 (64.6, 100)	98.5 (92.0, 99.7)	32.1%
Linezolid	0	0	0	0	0	0	0	87	3	90	NA	100 (95.9, 100)	NA	100 (95.8, 100)	3.3%
Bedaquiline	0	0	0	0	0	0	0	99	10	109	NA	100 (96.6, 100)	NA	100 (96.3, 100)	9.2%
Clofazimine	0	0	0	0	0	0	0	99	10	109	NA	100 (96.6, 100)	NA	100 (96.3, 100)	9.2%
Total	142	3	10	9	164	5	10	980	99	1094	88.4 (82.6, 92.5)	98.6 (97.7, 99.2)	93.5 (88.5, 96.5)	98.5 (97.5, 99.1)	8.7%

Refer to footnotes of Table 3 for details and abbreviations.

Although patient sputa were (presumably) all smear positive as per study design, the rate of successful Deeplex Myc-TB sequence analysis versus microscopic grade was not investigated in the Djibouti survey. This parameter was therefore evaluated on a set of 1494 direct sputum samples from a nationwide survey conducted in the Democratic Republic of Congo [22]. Yield of culture from CPC-stored sputum largely suffered from transport delays, whereas ethanol-preserved sputum samples were kept at room temperature for subsequent DNA extraction using a modified Maxwell 16 Low Elution Volume DNA Purification system [22]. Therefore, culture-free Deeplex Myc-TB testing was used mostly as a stand-alone assay for extensive pDST in TB patients, after their inclusion based on Ziehl–Neelsen smear positivity and positive *M. tuberculosis* detection on Xpert MTB/RIF.

Of the 1143 sputum samples with available microscopic examination data, mean pan-target read depth exceeded 1000x for samples graded 1+, 2+ and 3+ although, as expected, the dispersion towards lower values was inversely correlated with

microscopic grading (Figure 4A and 4B, Supplementary Table S14). Broadly similar read depths were also observed for samples that were graded negative (n=16 only, as per the standard survey design normally enrolling smear-positive TB patients only) or without reported grading (n=351). Despite the indeterminate bacterial loads in one third of the 1494 samples, MTBC was identified in 1258 (84.2%) of them. Of the 19,422 expected phenotypes in the 1494 samples, 73.5% (14,277) were automatically predicted (80.7%-82.4% for the four first-line drugs), based on detected resistance mutations, or absence of both resistance and uncharacterized mutations with minimal 5x read depth over $\geq 95\%$ of the reference targets. Uncharacterized mutations were detected for an additional 5.9% (n=1137), and 625 additional susceptible phenotypes (3.2%) could be predicted after verification of effective minimal coverage of all resistance positions in the corresponding target. When distinguished by microscopic grade, the proportion of predicted phenotypes in samples without microscopy results (81.1%) was actually higher than for samples that were graded 0+ (67.8%), 1+ (69.4%), 2+ (69.5%) and 3+ (76.3%).

Phenotypes for amino-glycosides, and to a lower extent, linezolid, were relatively less frequently predicted, as a reflection of comparatively lower average read depths on *rrl* and *rrl* rDNA targets (Supplementary Table S14), expectedly due to competing commensal rDNA reads. However, such competition does not detectably affect specific calling of variants in samples with well covered targets, as seen from the high degree of concordance with variants detected by WGS analysis on culture in the Djibouti dataset (except for minority variants only detected by deep sequencing that are likely true in most cases, Supplementary Note S6), and from full agreement between predictions of susceptible phenotypes for amino-glycosides and linezolid with those made by WGS analysis in the same dataset (Table 4).

DISCUSSION

Based on a large dataset, our results show a high degree of accuracy of the Deeplex Myc-TB assay for extensive prediction of both susceptibility and resistance to anti-TB drugs with an efficiency close to WGS, directly achievable at least from acid-fast bacillus smear positive clinical specimens. In contrast, susceptibility in particular cannot be reliably inferred from a negative result with conventional molecular tests, because of the limited set of resistance-conferring mutations covered [14].

Importantly, most of the residual predictions of Deeplex Myc-TB discordant with susceptible or resistant phenotypes in the tested set of 429 MTBC isolates, involved pyrazinamide, ethambutol, and ethionamide, for which pDST is an imperfect standard [3,31]. Likewise, for isoniazid or rifampicin, the sole discrepant resistant predictions by Deeplex Myc-TB all involved low-level resistance variants, frequently missed by liquid pDST, although they are critical to capture to avoid unfavorable treatment outcome or relapse [3,32,33]. For these reasons, sequencing of relevant genes is now proposed as a reference, at least for rifampicin, pyrazinamide, ethambutol, and ethionamide [31]. Deeplex Myc-TB can thus be considered as outperforming the pDST standard for these drugs.

Crucially in this respect, the agreement with WGS analysis was almost complete in both the in silico and experimental datasets. All heteroresistant calls detected by both methods in the set of 429 MTBC strains were concordant with resistance phenotypes. The sole heteroresistant variant exclusively detected (at 4.9%) - in an ethambutol susceptible phenotype - by deeper sequencing with Deeplex Myc-TB involved a *embB* M306V variant. Variants in this *embB* 306 codon (as well as codons 354, 406, and 497) are assumed to be resistance mutations, regardless of the pDST result obtained [36]. Likewise, most of the resistance predictions made only by direct

Deeplex Myc-TB analysis on sputa from the Djibouti survey were due to resistance minority variants, undetected by MTBSeq/PhyResSE/Mykrobe in WGS data likely due to insufficient read depths and/or culture bias eliminating potentially less fit (resistant) subpopulations [37] before subjection to WGS.

The optimal read depth-dependent limit of 3% for detecting minority populations with this Deeplex Myc-TB version was defined to secure true variant calls, after systematic analysis of sequence noise levels across target positions. This limit is substantially better than those of conventional molecular tests [38], although not as sensitive as the 0.1-1% limit claimed for the TGen deep sequencing assay [10,39]. While we recently developed an enhanced detection mode capable of reliably detecting minority variants down to 1%, this can only be reached on hyper-covered target portions. Going below this threshold results in an unacceptably higher rate of false positive base calls, affecting the overall specificity of such assays (data not shown). A genetic cutoff around this value seems reasonable, as old studies using the phenotypic proportion method showed that therapeutic success was unlikely above 1% of growing drug resistant bacilli [40]. However, the correspondence between phenotypic and genetic estimates could be influenced by the uncertainty of phenotypic proportions given the clumping nature of mycobacteria, and/or fitness cost of some resistance mutations resulting in underrepresentation of the cultured resistant subpopulation [37]. More studies are needed to address this important question.

Despite its large dataset, this study has limitations. The reference collection of 429 strains included relatively few resistant phenotypes for the second-line drugs investigated (none for bedaquiline and linezolid). Nevertheless, as also partly suggested from our *in-silico* analysis, the Deeplex Myc-TB targets and mutation

catalog comprise the main or even the exclusively established genomic targets and most determinants of resistance to these drugs in clinical isolates (e.g. *gyrA* and *gyrB* quinolone resistance determining regions for the fluoroquinolones). pDST data were not available for clinical specimens from Djibouti and DRC, as pDST is not routinely performed on all newly diagnosed TB patients in such resource-limited countries, and because of the difficulty to re-culture samples even with preservatives added upon storage and transportation. However, our results showed closely matching phenotype predictions between Deeplex Myc-TB and the ultimate genotypic reference, i.e. WGS analysis, on the Djibouti dataset. Finally, the number of tested smear-negative samples clearly identified as such in the DRC dataset was limited. Nevertheless, the established limit of detection of 100-1000 extracted genome copies indicates that the test can be applied on all smear-positive and at least part of smear-negative samples with any reasonably efficient DNA extraction method. Consistently, the similar mean read depths obtained on the 1+-, 2+- and 3+-graded specimens from the DRC survey also suggest a limit of detection equal or below 1+ (corresponding to 5000-10,000 genomes/ml of sample) with the DNA extraction conditions used, accounting also for the fact that the equivalent of about one tenth of the available 1-5ml of the DRC samples was generally used for DNA extraction and testing. Further consistent with such analytical sensitivity, in a recent study using QIAamp DNA Mini Kits for DNA extraction, complete Deeplex Myc-TB phenotypic predictions could be made from all 37 smear positive (including 5 scanty) and 2 smear negative samples, in a pilot series of 50 clinical specimens [41].

In conclusion, the results of this extensive evaluation demonstrate the potential of the Deeplex Myc-TB assay to reliably guide personalized TB treatment, from culture or directly from clinical specimens depending on their bacterial loads. As the test can

also be used on iSeq100 and MiniSeq, in addition to the MiSeq and NextSeq platforms used here, the scalability of throughputs, with optimal batches of 16 (iSeq100) to 384 tests/run (NextSeq) (including 3 controls/run), can cover the needs of many clinical laboratories at local/regional or nationally centralized levels. Its use might be particularly cost-effective upon positive MTBC detection (with or without rifampicin resistance) with a more sensitive rapid triage test such as GeneXpert MTB/RIF. Particularly when used on sputum, this assay will significantly reduce the total turnaround for generating extended DST reports. Culturing could potentially be restricted to samples without conclusive Deeplex Myc-TB results, and phenotypic DST potentially limited to drugs for which uncharacterized mutations (instead of resistance, benign or no mutation) are detected in the relevant genes, as proposed for WGS-based phenotype predictions from culture [3], or for the new/repurposed drugs bedaquiline, clofazimine and delamanid/pretomanid, for which resistance mutations are poorly characterized (*Rv0678* for bedaquiline/clofazimine) or not (yet) covered by the assay (*atpE* and *pepQ* for bedaquiline and all (candidate) genes for delamanid and pretomanid). Planned improvements include addition of such extra targets.

ACKNOWLEDGEMENTS

Mathilde Mairey (Genoscreen) is thanked for valuable technical help.

Parts of this work have been supported by the European Union PathoNGen-Trace project (FP7- 278864), and by the World Health Organization (for the TB drug resistance survey conducted in the Democratic Republic of Congo). BJ and LR were supported by European Research Council (ERC) starting grant "INTERRUPTB", Grant agreement 311725.

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LEGENDS

Figure 1. Deeplex Myc-TB results identifying a pre-XDR MTBC strain in a sputum DNA sample collected in a TB drug resistance survey conducted in the Democratic Republic of Congo. Information on *hsp65* best match-based identification, spoligotype (in this case, not yet known to the SITVIT database) and phylogenetic SNP-based identification of MTBC lineage is shown in the center of circle. Information on drug susceptibility and drug resistance predictions for 13 anti-TB drugs/drug classes is as follows. Target gene regions are grouped within sectors in a circular map according to the anti-tuberculous drug resistance with which they are associated. Sectors in red and green indicate targets in which resistance-associated mutations or either no mutation or only mutations not associated with resistance (shown in grey) are detected, resulting in predictions of resistant or susceptible phenotypes, respectively. Blue sectors refer to regions where as yet uncharacterized mutations are detected. Green lines above gene names represent the reference sequences with coverage breadth above 95%. Limit of detection (LOD) of heteroresistance (reflected by subpopulations of reads bearing a mutation) depends on the read depth at mutation position and is shown either as grey (LOD 3%) or orange zones (LOD >3%-80%) above reference sequences. Here, LOD is >3% at the end of a few targets only and over two *rrs* regions with usual lower coverage. *RIF: rifampicin, INH: isoniazid, PZA: pyrazinamide, EMB: ethambutol, SM: streptomycin, FQ: Fluoroquinolones, KAN: kanamycin, AMI: amikacin, CAP: capreomycin, ETH: ethionamide, LIN: linezolid, BDQ: bedaquiline, CFZ: clofazimine, SIT: spoligotype international type.

Figure 2. LOD of Deeplex Myc-TB for resistance variant detection. (Top) Read depth at resistance-associated Deeplex Myc-TB targets versus the number of input

genomes. Median values as well as 25-75% quartiles are shown. (Bottom) For each dilution level with 10, 10², 10³, 10⁴ genome copies, LOD was measured as the fraction of detected (green) or undetected (grey) resistance variants in total sets of 36 (near-) fixed (95-100%) and 16 minority mutations (5% frequency), spread across 4 independent replicated tests of 4 different MTBC genomic DNA extracts.

Figure 3. Venn diagrams representing the agreement between resistant phenotypes identified by 4 *M. tuberculosis* resistance and susceptibility prediction tools: Deeplex Myc-TB, MTBSeq, Mykrobe and PhyResSE. The numbers of resistant phenotypes respectively predicted by Deeplex Myc-TB analysis on 109 sputum samples from Djibouti and other analysis tools fed with WGS data from corresponding cultures are shown. (1) Two rifampicin-resistance phenotypes predicted by MTBSeq and PhyResSE and/or Mykrobe based on *rpoB* S431T and D435V, reflecting probable WGS or culture contaminations (see text). (2) Seven pyrazinamide-resistance phenotypes predicted for “*M. canettii*”-containing cultures by MTBSeq and Mykrobe based on *panD* M117T and *pncA* A46A, respectively. (3) One pyrazinamide-resistance phenotype predicted by MTBSeq based on *pncA* D136G. (4) Eleven resistant phenotypes predicted by Deeplex Myc-TB based on 10 minority variants (3-12%) and one *ethA* frameshift-causing indel. (5) Two streptomycin-resistance phenotypes predicted by Deeplex Myc-TB and Mykrobe based on *gidB* G69D. (6) Two ethambutol-resistance phenotypes predicted by Deeplex Myc-TB and PhyResSE based on *embB* S297A and Y319S.

Figure 4. Log read depth obtained by direct Deeplex Myc-TB testing of DNA extracted from clinical specimens collected in a TB drug resistance survey conducted in the Democratic Republic of Congo. (A) Log read depth at each drug resistance-associated Deeplex Myc-TB target on the total set of 1494 sputum samples. (B) Log

read depth at Deeplex Myc-TB targets according to microscopy gradings of 1143 sputum samples with available microscopic examination data. Median values as well as 25-75% quartiles are shown.

Supplementary Table S1. Summary of the datasets used in this study.

Supplementary Table S2. Identification of nontuberculous mycobacteria both by *hsp65*, *rrl* and/or *rrs* sequencing (Deeplex Myc-TB) and *rpoB*, 16S rDNA sequencing and phenotypic profiling (Institute of Tropical Medicine, ITM). The ID Code compares both identification results, as follows: FM-A: match at (subs-)species level (incl. synonymous (sub)species according to Tortoli *et al.* Infect Genet Evol 2019;75:103983); FM-B: match at (sub-)species level with clonal mix identified by Deeplex; FM-C: Match at complex level; M-D: Match at complex level, sublevel provided by Deeplex; M-E: Match at complex level, sublevel provided by the ITM; M-F: Match at complex level, different subspecies; PM: Partial match, mixed species identified by Deeplex; NIDx: No identification by Deeplex; NM: No match, different species; NIREF: No identification beyond genus level by the ITM.

Supplementary Table S3. Capture of the 120 resistance-determinants derived from the original training-set of 2099 MTBC genomes and their associated resistance phenotypes in Walker *et al.* [3], by the catalogue of resistance determinants in the Deeplex Myc-TB targets. The capture rate was calculated by summing the number of resistance determinants and their associated resistance phenotypes captured by Deeplex Myc-TB relative to the total numbers of resistance determinants and their associated resistance phenotypes detected by WGS in Walker *et al.* [3]. Mutations are annotated according to nucleotide and codon positions in reference genes of *M. tuberculosis* H37Rv. The *rpsA*_A440T mutation was not taken into account in the calculations as it is assumed to be redundant to *pncA* H57D in pyrazinamide

resistance phenotypes in *M. bovis* isolates. The numbers of susceptible phenotypes from the original publication are shown for visualizing the penetrance of the mutations with resistance phenotypes in the original dataset. In the original publication, each mutation can be counted more than once if it was characterized as a resistance-determinant to more than one drug.

Supplementary Table S4. Capture, by the catalogue of resistance determinants in the Deeplex Myc-TB targets, of mutations characterized in the training-set as resistance-determinants in Walker *et al.* [3] and retrieved in the validation-set of 1552 MTBC genomes of Walker *et al.* [3], together with their associated resistance phenotypes. The capture rate was calculated by summing the numbers of resistance determinants and their associated resistance phenotypes captured by Deeplex Myc-TB relative to the total numbers of resistance determinants and their associated resistance phenotypes detected by WGS in Walker *et al.* [3]. Mutations are annotated according to nucleotide and codon positions in reference genes of *M. tuberculosis* H37Rv. The *rpsA*_A440T mutation was not taken into account in these calculations as it is assumed to be redundant with *pncA* H57D in pyrazinamide resistance phenotypes in *M. bovis* isolates. The number of susceptible phenotypes from the original publication are shown for visualizing the penetrance of the mutations with resistance phenotypes in the original dataset.

Supplementary Table S5. SNPs detected in the 429 samples TDR and Sciensano-Belgian National TB Reference Center collections. Out of 2403 total variants, 54 were identified by Deeplex Myc-TB and not by WGS analysis with an initial frequency threshold of 85% for variant calling (2.2%, Detection=Y in Deeplex Myc-TB and Detection=N in WGS). All were non-fixed/minority variants. Of these 54 variants initially missed by WGS, 24 were detected by analysis under low frequency mode

(see variants with asterisks) and 30 were left completely undetected even under these conditions. Mutations are annotated according to nucleotide and codon positions in reference genes of *M. tuberculosis* H37Rv.

Supplementary Table S6. Phenotypes and genotypes of the 429 samples from the TDR and Sciensano-Belgian National TB Reference Center collections. Phenotypes (in column pDST-Deeplex) are presented in the form X-Y where X is the phenotype found using pDST and Y is the phenotype predicted by Deeplex Myc-TB (R for resistant, S for susceptible, U for uncharacterized). Genotypes are described per antibiotic and per Deeplex Myc-TB target. Mutations are in the format [gene: mutation (% call, variant type)]. They are displayed, in coding regions, as amino-acid substitutions while indels, or mutations in non-coding regions, are displayed as nucleotide substitutions (with gene position). Variant type can be R ("resistant"), S ("susceptible") and U ("uncharacterized"). All mutations were detected by both Deeplex Myc-TB and WGS except those only detected by Deeplex Myc-TB as described in Table S5. *RIF (Rifampicin), INH (Isoniazid), PZA (pyrazinamide), EMB (ethambutol), SM (streptomycin), FQ (fluoroquinolones), KAN (kanamycin), AMI (amikacin), CAP (capreomycin), ETH (ethionamide), LIN (linezolid). SBN TB Ref Center is the Sciensano-Belgian National TB Reference Center. The BCCM is the Belgian Coordinated Collections of Microorganisms (Mycobacteria) in Antwerp, Belgium.

Supplementary Table S7. Phenotype predictions by Deeplex Myc-TB analysis of DNA from 213 reference isolates from Sciensano-Belgian National TB Reference Center versus phenotype predictions by pDST performed by using liquid culture. For this strain collection subset, pDST was routinely done using the BACTEC MGIT960 system for first-line drugs, and the radiometric BACTEC 460 TB for second-line drugs

(see Methods). Resistance to streptomycin, kanamycin, capreomycin, bedaquiline and clofazimine was not assayed by pDST and is therefore not shown here.

Supplementary Table S8. Phenotype predictions by Deeplex Myc-TB analysis of DNA from 216 reference isolates from the WHO-TDR collection (BCCM) versus phenotype predictions by pDST performed by using solid culture. For this strain collection subset, pDST was performed by using the proportion method on Löwenstein-Jensen or Middlebrook 7H11 agar medium, for first- or second-line drugs, respectively (see Methods). Resistance to pyrazinamide, amikacin, linezolid, bedaquiline and clofazimine was not assayed by pDST and is therefore not shown here.

Supplementary Table S9. Variants detected in samples with phenotype R but with S or U predictions by Deeplex Myc-TB (R-S or R-U in pDST-Deeplex, Table S6), in regions that are not covered by the assay: RIF (extended *rpoB*), INH (*mshA*, *furA*, *ndh*, extended *fabG1*, extended *katG*), PZA (*rpsA*, *panD*, extended *pncA*), EMB (*embA*, *embC*, *embR*, *iniA*, *iniC*, extended *embB*), SM (extended *gidB*, *rpsL* and *rrs*), FQ (extended *gyrA* and *gyrB*), KAN (extended *eis* and *rrs*) & ETH (*ethR*, *Rv0565c*, *Rv3083*). Extended regions are full coding sequence and promoter regions of genes from which only (most) critical regions are covered by Deeplex Myc-TB. Mutations are annotated according to nucleotide and codon positions in reference genes of *M. tuberculosis* H37Rv.

Supplementary Table S10. Specificity and sensitivity of Deeplex Myc-TB in comparison with pDST, based on 429 strain DNA samples from the TDR and Sciensano-Belgian National TB Reference Center collections, using a predicted susceptibility to isoniazid as predictor of susceptibility to other first-line drugs, for which corresponding gene targets are devoid of drug resistance mutations but

contain uncharacterized mutations.

Supplementary Table S11. SNPs detected in 109 samples from the Djibouti collection. Out of 752 total variants, 74 were identified by Deeplex Myc-TB and not by WGS analysis with an initial frequency threshold of 85% for variant calling (9.84%, Detection=Y in Deeplex Myc-TB and Detection=N in WGS) and 1 variant was detected by WGS only (0.13%, Detection=N in Deeplex Myc-TB and Detection=Y in WGS). All were non-fixed/minority variants. Of the 74 variants initially missed by WGS analysis at 85% frequency, 15 were detected by analysis under low frequency mode (see variants with asterisks) and 59 were completely undetected even under these conditions.

Supplementary Table S12. Phenotype predictions by direct Deeplex Myc-TB analysis of DNA from 109 clinical specimens versus phenotype predictions by PhyResSE with WGS data from culture. Deeplex Myc-TB sequencing and WGS data originate from a report of a national TB drug resistance survey conducted in Djibouti [13]. Resistance to bedaquiline/clofazimine is not predicted by PhyResSE and is therefore not shown here.

Supplementary Table S13. Phenotype predictions by direct Deeplex Myc-TB analysis of DNA from 109 clinical specimens versus phenotype predictions by Mykrobe with WGS data from culture. Deeplex Myc-TB sequencing and WGS data originate from a report of a national TB drug resistance survey conducted in Djibouti [13]. Resistance to ethionamide, linezolid and bedaquiline/clofazimine is not predicted by Mykrobe and is therefore not shown here.

Supplementary Table S14. Average read depth per Deeplex Myc-TB target in the 1494 sputum samples from the DRC collection. Microscopy grading is also provided when available (1143 samples).

Supplementary Table S15. Average read depth per Deeplex Myc-TB target in the 429 DNA strain samples from the TDR and Sciensano-Belgian National TB Reference Center collections. *Rv0678* and *ahpC* were not yet targets of the Deeplex Myc-TB kit version used at the time of analysis of the Sciensano-Belgian National TB Reference Center collection. SBN TB Ref Center is the Sciensano-Belgian National TB Reference Center. The BCCM is the Belgian Coordinated Collections of Microorganisms (Mycobacteria) in Antwerp, Belgium.

Supplementary Figure S1. Limit of detection (LOD) of Deeplex Myc-TB for MTBC identification. (Top) Read depth at *hsp65* versus the number of input genomes. Median values as well as 25-75% quartiles are shown. (Bottom) For each dilution level with 10, 10², 10³, 10⁴ genome copies, LOD was measured as the fraction with (green) or without (grey) correct identification among 16 tests, corresponding to four independent amplification replicates and sequencing rounds of 4 MTBC genomic DNA extracts.

Supplementary Figure S2. Limit of detection (LOD) of Deeplex Myc-TB for NTM identification. (Top) Read depth at *hsp65* versus the number of input genomes of a *M. intracellulare* strain. Median values as well as 25-75% quartiles are shown. (Bottom) LOD was measured as the fraction with (green) or without (grey) correct identification in three independent replicated analyses of 10, 10², 10³, 10⁴ *M. intracellulare* genome copies.

Supplementary Figure S3. Identification of nontuberculous mycobacterial species by Deeplex Myc-TB versus reference identification. Deeplex Myc-TB results were based on *hsp65* best match analysis, complemented by specific SNP detection in 16S (*rrs*) and 23S rDNA (*rrl*) targets for *M. kansasii* and *M. chelonae* strains, while reference identification was based on *rpoB* and/or 16S rDNA Sanger sequencing

results, phenotypic profiling and/or type strain status. The number of isolates studied per taxon (complex, species or subspecies) is proportional to associated circle sizes. FM-A, FM-C: Full match at same taxonomic level (complex, species or subspecies), FM-B: Full match at (sub)species level but with a clonal mix identified by Deeplex, M-D: Match at complex level, sublevel provided by Deeplex, M-E: Match at complex level, sublevel provided by the reference, M-F: Match at complex level, different subspecies, PM: Partial match, several possible species identified by Deeplex, NM: No match. Non-tuberculosis mycobacterial taxa are ordered phylogenetically, according Tortoli et al. Infect Genet Evol 2019;75:103983.

Supplementary Figure S4. Deeplex Myc-TB detecting co-existing *hsp65* reads of *Mycobacterium tuberculosis* complex and *Mycobacterium gastri* strains in DNA from an individual sputum sample collected in a TB drug resistance survey conducted in Djibouti. The respective percentages of *hsp65* reads detected for each species are shown under “*hsp65*-based identification best-match”. Explanations in the legend of Figure 2 in the main text apply for other parts of this figure.

Supplementary Figure S5. Log read depth obtained at drug resistance-associated Deeplex Myc-TB targets in 429 DNA strain samples from the TDR and the Sciensano-Belgian National TB Reference Center collections. Median values as well as 25-75% quartiles are shown.

Supplementary Figure S6. Deeplex Myc-TB results obtained using 10^2 versus 10^4 *M. tuberculosis* genome copies of sample TB-TDR0014 from the TDR collection. A red arrow indicates an erratically detected, false positive, synonymous low frequency variant (5.2%) *pncA* Y34Y at 10^2 genomes. This variant was detected only in this experiment. This reflects very sporadic emergence over filtered noise level of low frequency amplification errors, sometimes seen with 10 or 10^2 genome copies.

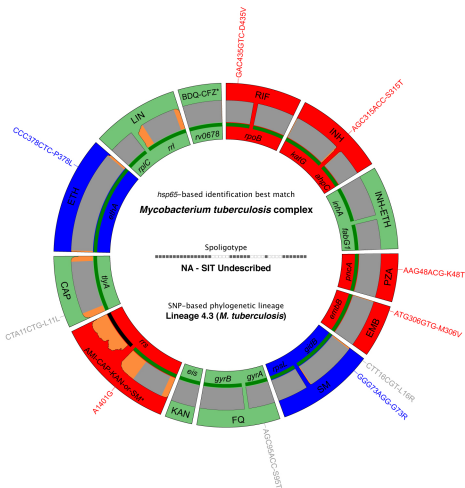


Figure 1. Deeplex Myc-TB results identifying a pre-XDR MTBC strain in a sputum DNA sample collected in a TB drug resistance survey conducted in the Democratic Republic of Congo. Information on *hsp65* best match-based species identification, spoligotype (in this case, not yet known to the SITVIT database) and phylogenetic SNP-based identification of MTBC lineage is shown in the center of the circle. Information on drug susceptibility and drug resistance predictions for 13 anti-TB drugs/drug classes is as follows. Target gene regions are grouped within sectors in a circular map according to the anti-tuberculous drug resistance with which they are associated. Sectors in red and green indicate targets in which resistance associated mutations, or either no mutation or only mutations not associated with resistance (shown in grey) are detected, resulting in predictions of resistant or susceptible phenotypes, respectively. Blue sectors refer to regions where as yet uncharacterized mutations are detected. Green lines above gene names represent the reference sequences with coverage breadth above 95%. Limits of detection (LOD) of heteroresistance (reflected by subpopulations of reads bearing a mutation), depends on the read depth at mutation position and is shown either in grey (LOD 3%) or orange zones (LOD >3%–80%). Here, LOD is >3% at the end of a few targets only and over two *rrs* regions with usual lower coverage. *AMI: amikacin, BDQ: bedaquiline, CAP: capreomycin, CFZ: clofazimine, EMB: ethambutol, ETH: ethionamide, FQ: fluoroquinolones, KAN: kanamycin, LIN: linezolid, INH: isoniazid, PZA: pyrazinamide, RIF: rifampin, SM: streptomycin, SIT: spoligotype international type.

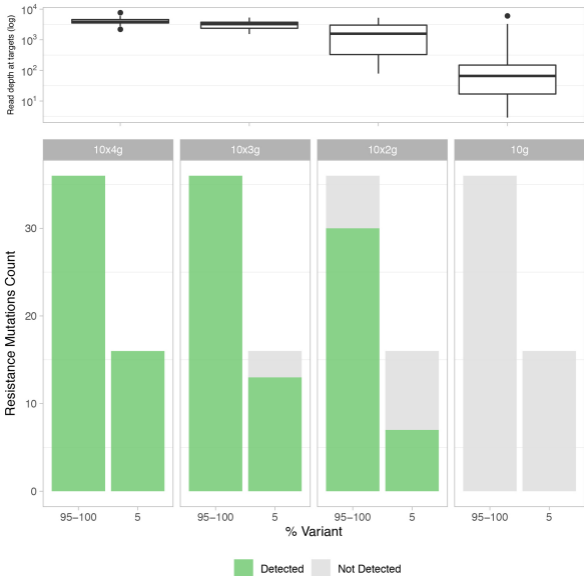


Figure 2. LOD of Deeplex Myc-TB for resistance variant detection. (Top) Read depth at resistance-associated Deeplex Myc-TB targets versus the number of input genomes. Median values as well as 25-75% quartiles are shown. (Bottom) For each dilution level with 10, 10², 10³, 10⁴ genome copies, LOD was measured as the fraction of detected (green) or undetected (grey) resistance variants in total sets of 36 (near-) fixed (95-100%) and 16 minority mutations (5% frequency), spread across 4 independent replicated tests of 4 different MTBC genomic DNA extracts.

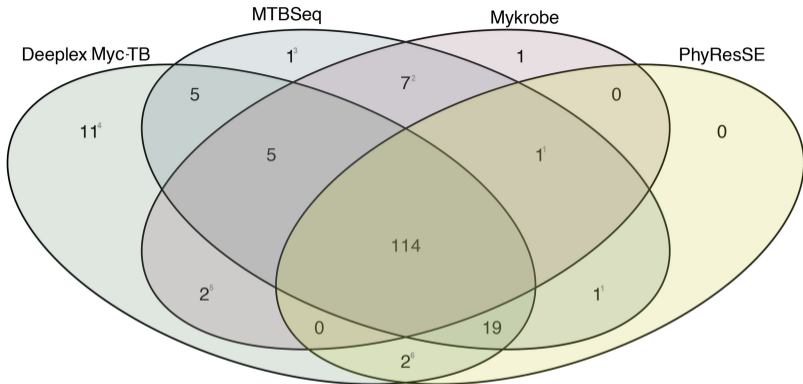


Figure 3. Venn diagram representing the agreement between resistant phenotypes identified by 4 *M. tuberculosis* resistance and susceptibility prediction tools:

Deeplex Myc-TB, MTBSeq, Mykrobe and PhyResSE. The numbers of resistant phenotypes respectively predicted by Deeplex Myc-TB analysis on 109 sputum samples from Djibouti and other analysis tools fed with WGS data from corresponding cultures are shown. (1) Two rifampicin-resistance phenotypes predicted by MTBSeq and PhyResSE and/or Mykrobe based on *rpoB* S431T and D435V, reflecting probable WGS or culture contaminations (see text). (2) Seven pyrazinamide-resistance phenotypes predicted for *M. canettii*-containing cultures by MTBSeq and Mykrobe based on *panD* M117T and *pncA* A46A, respectively. (3) One pyrazinamide-resistance phenotype predicted by MTBSeq based on *pncA* D136G. (4) Eleven resistant phenotypes predicted by Deeplex Myc-TB based on 10 minority variants (3-12%) and one *ethA* frameshift-causing indel. (5) Two streptomycin-resistance phenotypes predicted by Deeplex Myc-TB and Mykrobe based on *gidB* G69D. (6) Two ethambutol-resistance phenotypes predicted by Deeplex Myc-TB and PhyResSE based on *embB* S297A and Y319S.

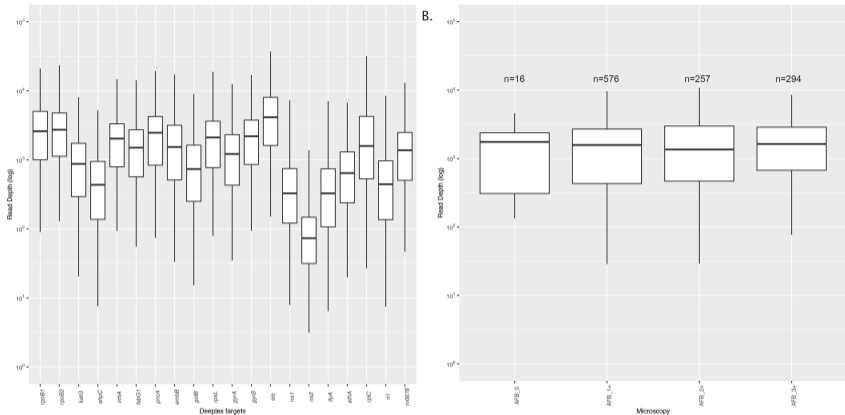


Figure 4. Log read depth obtained by direct Deeplex Myc-TB testing of DNA extracted from clinical specimens collected in a TB drug resistance survey conducted in the Democratic Republic of Congo. (A) Log read depth at each drug resistance-associated Deeplex Myc-TB target on the total set of 1494 sputum samples. (B) Log read depth at Deeplex Myc-TB targets according to microscopy gradings of 1143 sputum samples with available microscopic examination data. Median values as well as 25-75% quartiles are shown.

Supplementary material	
<i>Paper title</i>	Deep amplicon sequencing for culture-free prediction of susceptibility or resistance to 13 anti-tuberculous drugs
<i>Authors</i>	Agathe Jouet, Cyril Gaudin, Nelly Badalato, Caroline Allix-Béguet, Stéphanie Duthoy, Alice Ferré, Maren Diels, Yannick Laurent, Sandy Contreras, Silke Feuerriegel, Stefan Niemann, Emmanuel André, Michel K Kaswa, Elisa Tagliani, Andrea Cabibbe, Vanessa Mathys, Daniela Cirillo, Bouke C de Jong, Leen Rigouts, Philip Supply
PMID	

S1. Limit of detection for MTBC or NTM identification

As Deeplex Myc-TB does not depend on a specific DNA extraction method, the assay's limit of detection for MTBC identification was estimated in terms of number of extracted MTBC genome copies by performing serial dilutions of purified, pre-quantified genomic DNA from three well-characterized MTBC strains, and a mixture of two strains at a 5-95% ratio. Four independent amplification replicates (each with a different kit lot) and sequencing rounds were performed for each dilution, yielding a total of 16 tests per dilution level (10, 10², 10³ and 10⁴ genomes). Based on *hsp65* sequence best match analysis, all samples were identified as *M. tuberculosis* at 10⁴ and 10³ genomes, 13/16 samples (81.2%) at 10² genomes while none were identified with 10 genomes (Supplementary Figure S1).

Likewise, all three independent replicated analyses of 10², 10³ and 10⁴ genomes of *M. intracellulare* resulted in the expected 100% identification based on *hsp65* best match analysis, while two of three replicates with 10 genomes generated this expected result (Supplementary Figure S2).

S2. Assay design

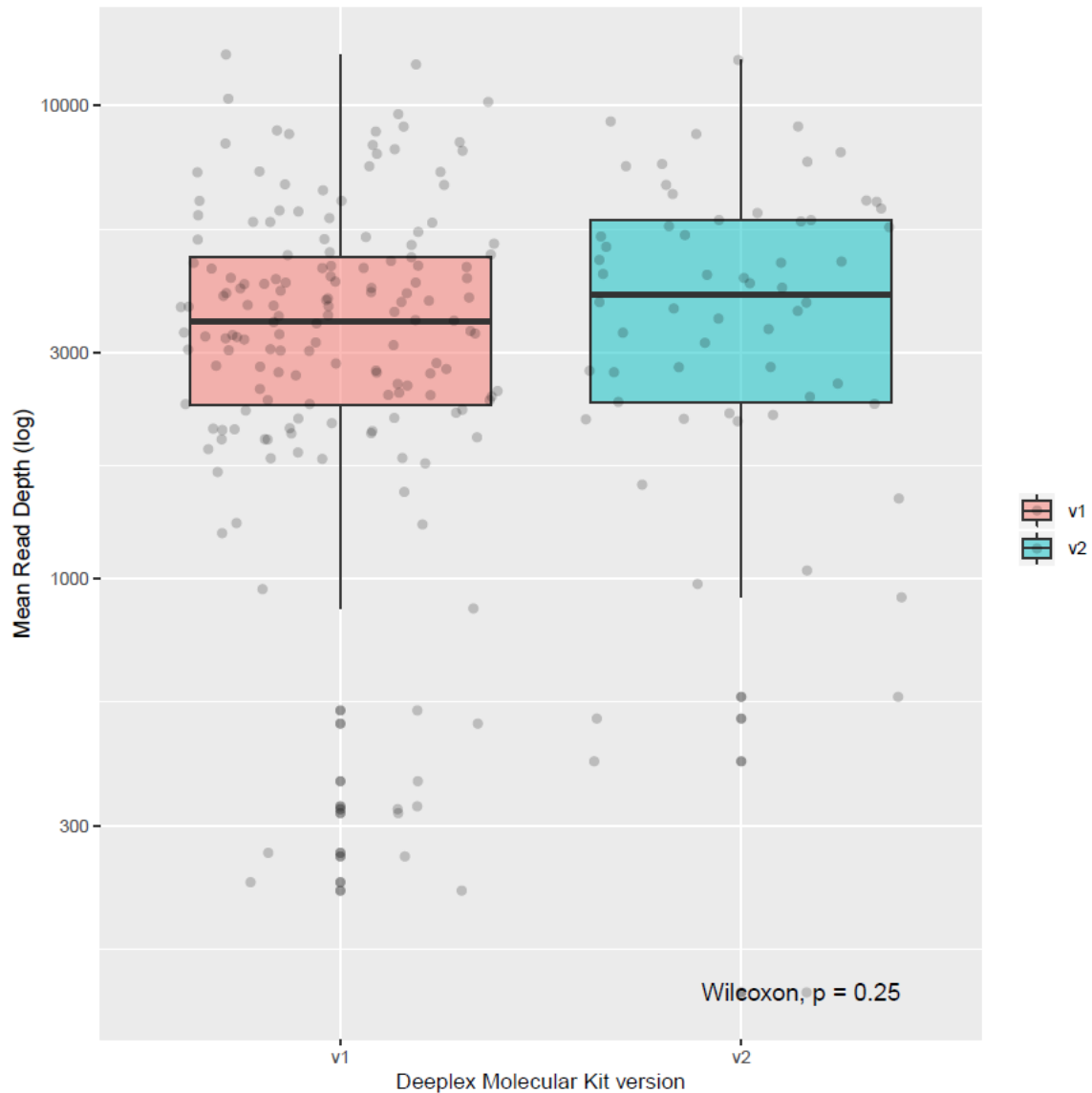
The assay is based on a single 24-plex PCR targeting 18 major genes associated with resistance to 13 first- and second-line anti-TB drugs/drug classes, amplified in 21 amplicons (Figure 1 and Table 1 in the main text). Two separate amplicons cover

rpoB codons 384-516 and 158-335, including not only the rifampicin resistance determining region but also other regions containing known rifampicin resistance mutations that are missed by rapid molecular tests (e.g. I491F [1,2] and V170F [3]). For genes such as *pncA* and *Rv0678*, associated with pyrazinamide and bedaquiline/clofazimine resistance respectively, where resistance mutations are known to arise across the entire corresponding sequences, complete coding sequences along with part of promoter regions are covered by a single amplicon. For *ethA*, associated with ethionamide resistance, two overlapping amplicons cover the gene and part of the promoter. A 401-bp segment of the *hsp65* gene is used as primary reference for mycobacterial species identification [4], the direct repeat region for spoligotype identification of MTBC strains [5], and an internal control sequence to control PCR inhibition.

After PCR and amplicon library preparation and sequencing, reads from FASTQ files uploaded to the Deeplex Myc-TB web application are automatically mapped on *M. tuberculosis* H37Rv reference sequences using Bowtie 2 [6]. Variants are called with a limit of 3% read proportion depending on coverage depth at the position (134x required at least), using proprietary scripts and filters. Detected variants are automatically associated with drug resistance or susceptibility, or phylogenetic SNPs (i.e. MTBC (sub)lineage-defining) by comparison with integrated reference variant databases (Table 2 in the main text). Priority is given to the collaborative, curated database ReSeqTB when detected variants are included in this database [7]. When not included in these databases, mutations are defined as uncharacterized. Such categorization for off-catalogue mutations, instead of explicit or implicit prediction of susceptibility as currently done by other tools [8–10], has been shown to be an effective flag to avoid some very major errors (missed resistance) and trigger

subsequent pDST for confirmation [11]. Mycobacterial species and spoligotype are identified using additional integrated databases, based on best-match against *hsp65* sequences from 167 mycobacterial species/subspecies derived from data from Dai et al. [4] and *M. kansasii* and *M. chelonae*-specific mutations in *rrs* and *rrl*, and “SIT” types from the global SITVIT database [12], respectively. The publication from Dai et al. was selected as a primary reference for NTM identification because the majority of the *hsp65* sequence entries were distinctively curated by validation with type strains or were supported by multiple GenBank records deposited by independent research groups.

For some datasets (i.e. obtained from isolates from Sciensano and the TDR collection, clinical specimens from Djibouti, and a subset of clinical specimens from the Democratic republic of Congo), a beta version of the Deeplex Myc-TB kit was used, which differed from the up-to-date version (including the targets described in Table 1 in the main text) by not yet including the clofazimine/bedaquiline resistance-associated target *Rv0678* and the *ahpC* promoter region including two rare isoniazid resistance-associated mutations, namely G-48A and C-57T. To verify that the coverage depths on the other targets did not differ between the two versions, the mean read depths on these targets on 6 replicates of the same *M. bovis* BCG positive control obtained either with the first or the second versions were compared. After verifying the normality of the data with Q-Q plots using R, no significant difference was found ($p > 0.05$) by using a non-parametric test (Wilcoxon) between the log mean read depths obtained with the first and the second versions (see the figure below).



S3. Mycobacterial species identification

For extensive testing of mycobacterial species identification, DNA extracts from 370 nontuberculous mycobacterial (NTM) strains from the Institute of Tropical Medicine of Antwerp (ITM, Belgium) were used, including 3 strains used in routine testing at the ITM, 30 type strains and 337 strains from the Belgian Culture Collection of Microorganisms (BCCM), maintained under quality control at ITM. These represented 73 different NTM species/species complexes, identified at the ITM based on a combination of phenotypic testing, *rpoB* and/or 16S rDNA Sanger sequencing. Multiple strains from the major clinically relevant taxa were purposely included to

represent a potential intraspecies diversity. After application of the assay on these DNA extracts, the Deeplex Myc-TB web application was used for automated best-match analysis using *hsp65* as a primary gene target, complemented by *rrs* and *rrl* sequence analysis for some mycobacterial species (Supplementary Table S2).

Out of these 370 strains, 292 strains were identified at (sub)species or species complex levels by both reference and Deeplex Myc-TB testing (Supplementary Figure S3, Supplementary Table S2). Of these, 194 (66.4%) were best-matched at the same taxonomic level by both methods, while 76 (26%) were matched at the same complex level, with a sublevel identification by Deeplex Myc-TB (n=20; essentially *M. intracellulare* complex strains), or the reference (n=40; essentially *M. avium* complex strains, relatively overrepresented in this dataset, which are not further identified as (*avium* or *hominisuis*) subspecies by Deeplex Myc-TB), or showing a different subspecies (n=16) across methods. Among the 194 cases concordant at the same taxonomic level, two distinct *hsp65* sequence variants both best matching a same species were detected by Deeplex Myc-TB in 13 strains, indicating underlying clonal complexity (e.g. in 3 *M. nonchromogenicum* and 2 *M. smegmatis* strains). In addition, 4 of 292 (1.4%) strains had a partial match either because they were best matched between two possible equidistant species (n=1), or detected in a mixture with another species (n=3). Only 18 (6.2%) strains had taxonomically discordant results between both methods, i.e. without full or partial match as above. These mostly consist of single discordant cases among otherwise partially/fully concordant strains for a species (e.g. *M. ulcerans*, n=1/13; *M. kansasii*, n=1/14), or species rarely involved in infections (e.g. *M. peregrinum*; n=2) [13,14].

To note, for residual isolates with discrepant subspecies within a matching complex (e.g. *M. intracellulare* vs *M. chimaera*), the correctness of the Deeplex Myc-TB

identification was actually often possible or probable. Indeed, in these cases, conflicting or ambiguous identifications were frequently seen between the *rpoB* and 16S rDNA reference probes, with one or the other partially or fully matching the Deeplex Myc-TB result (Supplementary Table S2). This held also true for some of few individual isolates (of otherwise well identified species) that were discordant even at complex level (e.g. *M. kansasii* vs *M. gastri*).

Of the remaining 78 strains out of the 370 analyzed in total, 5 had no identification by either method while the others were identified to (sub)species/complex level by Deeplex Myc-TB (n=28) or the reference (n=45) only. The latter mostly consisted of strains from species of rare clinical prevalence (including 5 other *M. peregrinum* or *M. septicum* strains), or reflect a minority of cases among strains otherwise partially/fully concordant for a species or complex (e.g. 6/16 *M. chelonae* complex, including 1/11 *M. chelonae subsp chelonae*). Only a few NTM species of relatively higher clinical significance had a majority of strains without Deeplex best matched species, including in particular some strains from the *M. terrae* complex: *M. arupense* (2/3 without best match), and *M. virginiense* (1/2; the other strain being best matched to *M. aurum*).

Altogether, these results thus indicate highly reliable identification of mycobacterial species by the Deeplex Myc-TB. The range of 167 mycobacterial (sub)species covered by the identification database, including virtually all clinically relevant ones, is substantively larger than that of conventional molecular tests, such as line probe assays covering 46 (sub)species in two separate assays [15], and is close to that of MALDI TOF mass spectrometry-based methods, such as Bruker's (Billerica, Massachusetts, USA) reportedly covering 178 (sub)species (<https://www.bruker.com/products/mass-spectrometry-and-separations/maldi->

[biotyper-for-microbiological-research/mycobacteria/overview-mycobacteria.html](https://www.bacterio.net/genus/mycobacterium)). We plan to further increment our identification database by first basing on the comprehensive, updated list of taxa with a validly published and correct name, included in the Prokaryotic names with Standing in Nomenclature (LSPN) database (<https://www.bacterio.net/genus/mycobacterium>). While the number of such taxa (excluding synonyms) as of August 2020 is 192 in this list, most of those not (yet) included in our database concern relatively novel species described by a single research group thus far, and based on very few or even just one strain(s) in most cases. As was done in the work from Dai et al. [4] (see above), we will prioritize further incorporation of new taxa that are described by independent research groups. Of final note, the detection of distinct subpopulations of variant *hsp65* reads within some samples of the NTM strain collection (Supplementary Table S2) as well as in some clinical specimens from Djibouti (see below and Supplementary Figure S4), indicate distinct co-existing species (NTM or NTM with MTBC) or species variants. Detection of co-infecting or co-colonizing mycobacterial species is important for correct interpretation of available pDST results and guidance of patient treatment [16].

S4. Deeplex Myc-TB phenotype prediction versus phenotypic testing

We compared the ability of both Deeplex Myc-TB and Illumina-based WGS analysis to detect variants, using a total of 429 strains. For this comparison, we used a state-of-the-art WGS analysis pipeline, validated for accurate SNP calling by using short read data sets derived from isolates with known complete genomes in accordance with recent guidelines [17,18]. Mean on-target coverage depth by Deeplex Myc-TB was 3,171x (SD=3691.7) (Supplementary Figure S5, Supplementary Table S15), with a minimum mean of 600x on *rrs2*, which largely exceeds the minimal read depth set

to reach the maximal limit of detection of 3% for minority variants (*i.e.* $\geq 134x$). Mean reference coverage breadth was 99% (SD=4.9). In comparison, mean coverage depth and breadth on the same targets by WGS was 71x (SD=70) and 98.9% (SD=6.2), respectively.

In total, 2403 variants were identified in the Deeplex Myc-TB targets (2323 SNPs, 65 deletions and 15 insertions), including 798 resistance-associated variants. Of all variants, 2349 (97.8%; including 2269 SNPs and all 80 indels), including 779 (97.6%) of the resistance variants, were detected by both Deeplex and our WGS analysis pipeline initially used with a common high-frequency threshold (85%) used for SNP calling. The remaining 54 (2.2%) SNPs were all variants with frequencies below this threshold (Supplementary Table S4). Of these, 34 (63.0%) were found as co-occurring with 1 to 7 other such variants within an individual isolate (involving resistance and/or phylogenetic variants in 26 cases), indicating mixed strains and/or combined heteroresistance and thus genuine genotypic subpopulations detected by targeted deep sequencing. Moreover, about half of the total 54 ($n=24$) were identified in WGS-derived reads when analyzed under low frequency detection mode, detected with similar ~5-80% frequencies as in Deeplex Myc-TB-derived reads in virtually all cases. The other half ($n=30$), undetected even under these conditions, concentrated those with lowest frequencies of ~3-10% only identified by targeted deep sequencing, as an expected reflection of the lower coverage depth obtained with WGS (Supplementary Table S4). Of these, only one (at a frequency of 4.9%) corresponded to a resistance-associated mutation (to ethambutol; see main text).

Of the 1583 susceptible phenotypes with prediction, only 41 (2.6%) were discordantly predicted as resistant. Among these, 28 (68.3%) involved ethambutol and *embB* mutations known to be associated with poor phenotypic reproducibility (*e.g.* 12

M306V, 7 M306I and 6 G406D [19], including two minority variants, one of which was not detected by WGS (4.9%). Six discordances consisted of indels (all confirmed by WGS) causing frameshifts in *ethA*, all mechanistically expected to cause ethionamide resistance due to defective drug activation, as a result of impaired encoding of the monooxygenase EthA [20]. Also meaningful in this respect, four other *ethA* frameshifts were found in ethionamide-resistant phenotypes, in line with notorious phenotypic variability for this drug [21]. The only three discordances for isoniazid involved *inhA* S94A (without *fabG1* C-15T) in two isolates and *ahpC* G-48A in one isolate, showing variable association with (low level) isoniazid resistance among different studies [7,19,22]. Likewise, the only three discordant predictions for rifampicin involved *rpoB* L452P, H445N and D435Y, all part of the so-called “disputed” mutations with low rifampicin resistance levels frequently missed by liquid pDST, yet associated with poor clinical outcome on rifampicin-based treatment [23,24]. Lastly, the *gidB* A138V mutation, found in one streptomycin-susceptible isolate and associated with streptomycin resistance by Deeplex Myc-TB, is also subject to conflicting association with streptomycin resistance [7,25].

S5. Interpretation of uncharacterized mutations conditional on isoniazid susceptibility results

Because susceptibility to isoniazid predicts susceptibility to other first-line drugs [26] and as described for WGS-based prediction [27], the overall predictability of the test was further improved when a predicted susceptibility to isoniazid was considered to predict susceptibility to other first-line drugs, even when uncharacterized mutations were present in gene targets relevant for these drugs. This reduced the proportions of uncharacterized predictions from 1.9-4.4% to 0.5-2.5% for rifampicin, ethambutol and pyrazinamide, with correct re-classification as susceptible phenotypes in 5, 3 and

1 cases, respectively (Supplementary Table S7). To note however, this also resulted in the incorrect prediction of one additional susceptible phenotype (i.e. for a resistant phenotype) for each of these drugs.

S6. Variant detection by Deeplex Myc-TB on clinical specimens versus WGS analysis

We compared variant detection and phenotype predictions based on available Deeplex Myc-TB sequencing data directly obtained from 109 clinical specimens from a nationwide survey conducted in Djibouti, versus analysis of WGS data obtained after culturing [28]. DNA was extracted from these sputum samples as per a protocol described in the Deeplex-MycTB user's manual, as follows. After decontamination by using N-acetyl-L-cysteine combined with sodium hydroxide and sodium citrate procedure, utilizing NACPAC™RED kit from AlphaTec (Vancouver, Washington state, USA), followed by treatment by NPC67 neutralizing buffer and resuspension in 0.5 ml – 1 ml of PRB pellet resuspension buffer, both from AlphaTec, 100 µl to 500 µl of the suspensions were transferred in a screw cap or safe-lock microfuge tube and heat inactivated at 95°C for 30 min in an oven. After subsequent centrifugation at 20,000 g for 30 min, the supernatant was removed and discarded, and 250 µl of 10 mM Tris-HCl pH 7.8 was added, followed by brief vortexing, incubation at 95°C for 15 min and brief spinning down to collect potential droplets in tube cap. The entire volume (avoiding the pellet) was transferred in a new tube containing molecular biology grade 0.15 mm zirconium beads (Next Advance, Troy, New York, USA) in 2ml microtubes, vortexed at least for 30s, using a benchtop vortex mixer at full speed, followed by brief spinning down and incubation at least for 30 min at -20°C. After thawing at room temperature, the supernatant was transferred in a new 1,5 ml microtube, by avoiding resuspension of beads. A DNA concentration step was then

performed by adding 1 μ l of 20 mg/ml glycogen solution (Sigma Aldrich, Saint-Louis, Missouri, USA) and mixing, 0.1 volume of 3 M sodium acetate at pH 5.2 and mixing, and 3 volume 100% pre-cooled ethanol, followed by vigorous vortexing for 10s, incubation at -20°C for 10 min, and centrifugation at 15,000 g for 20 min. After discarding of the supernatant, 600 μ l of freshly prepared, pre-cooled 70% ethanol were added, followed by centrifugation at 15,000 g for 5 min. After discarding the supernatant, the pellet was air dried for 15 min and resuspended in 20 μ l of sterile water.

Overall, 693 of 752 total variants (92.2%) were detected by both Deeplex Myc-TB and our WGS pipeline used under low frequency detection mode. Apart from one fixed *pncA* SNP not detected by Deeplex Myc-TB, 59 SNPs were completely missing from WGS reads even when analyzed under low frequency detection mode. All latter SNPs were minority variants with frequencies from 3 to 25% in most cases, clustering in 20 samples. Of these 59, 45 were, or co-occurred at similar low frequencies with, resistance and/or phylogenetic variants within a sample, suggesting likely true variant calls missed by a generally (much) lower read depth by WGS (Supplementary Table S11). Of the remaining 14 SNPs, some could possibly reflect Deeplex Myc-TB amplification of false positive SNPs occasionally seen in test samples with low genome copy numbers (Supplementary Figure S6). However, when occurring, such sporadic effect typically affects only one to few uncharacterized or synonymous minority SNPs – representing the vast majority of possible nucleotide changes across all targets- at frequencies close to the 3% limit of detection (Supplementary Figure S6).

Of note, within some of these clinical specimens from Djibouti, Deeplex Myc-TB detected distinct subpopulations of variant *hsp65* reads (Supplementary Figure S4),

indicating distinct co-existing species (NTM with MTBC). Detection of such NTM/MTBC co-infections has diagnostic and clinical implications [29].

Moreover, potential/probable mixed infections with *M. tuberculosis* and “*M. canettii*” or strains of distinct MTBC lineages strains were detected by Deeplex MycTB in 8/109 (7.3%) sputum samples from Djibouti (and 37/1494 (2.3%) sputum samples from DRC), based on mixed phylogenetic SNP calls, possibly supplemented by the observation of a biphasic distribution of read depths on spoligotype spacers. This detection of mixed infections is important for epidemiological inferences, and may be as well of clinical relevance, as mixed infections have been associated with poor tuberculosis treatment outcomes, also independently of heteroresistance [30].

S7. Deeplex Myc-TB on clinical specimens vs MTBSeq-based predictions

The sensitivity of 93.5% for rifampicin of Deeplex Myc-TB versus MTBSeq on the Djibouti dataset resulted from two resistance predictions by MTBSeq (also by PhyResSE and Mykrobe; Figure 3 in main text) based on the detection of two minor variants in *rpoB* (S431T at 42.7%, and D435V at 25.9%) that co-occurred with one or more minor variants in other genes (Deeplex Myc-TB targets as well as other genes) sequenced by WGS. None of these minor variants were detected in the two corresponding sputa analyzed by Deeplex Myc-TB (even before filtering of the sequence data) despite high coverage depths at these positions, suggesting genotypic heterogeneity or contamination, introduced or amplified during subsequent culturing or WGS processing.

Fifteen of the 995 (1.5%) phenotypes predicted as susceptible by MTBSeq in the Djibouti dataset (excluding uncharacterized phenotypes by Deeplex Myc-TB) were identified as resistant by Deeplex Myc-TB (Table 4 in main text). Of these, 10 (66.6%) discordances were due to minority resistance-associated variants at 3.3-

12.8% detected in sputa by Deeplex Myc-TB, but missed by MTBSeq, PhyResSE and Mykrobe (Figure 3; *katG* S315T (n=2); *embB* G406A and co-occurring G406A + P404S; *pncA* D8G, H57D, H71Y; *gyrA* D94A, and *rrs* A514C (n=2)). The five remaining discordant resistance predictions resulted from divergent variant interpretation, involving one *ethA* frameshift-causing indel (mechanistically expected to cause ethionamide resistance, see above), two debated *embB* mutations (S297A, Y319S [35]) and the *gidB* G69D mutation (n=2, associated with low-level streptomycin resistance [25]), which were associated with resistance by both Deeplex Myc-TB and PhyResSE and/or Mykrobe, but not by MTBSeq

S8. Deeplex Myc-TB on clinical specimens vs PhyResSE and Mykrobe WGS-based predictions

With the 950 predicted phenotypes by Deeplex Myc-TB (137 resistant, 813 susceptible), the mean sensitivity and specificity versus available PhyResSE predictions were 98.5% and 97.2% (Supplementary Table S12). Ninety additional phenotypes (8.7%) were not predicted by Deeplex Myc-TB due to the presence of phenotypically uncharacterized mutations. Agreement on resistance prediction was 100% for all applicable drugs, except for rifampicin (93.5%). The latter score resulted from two PhyResSE resistance predictions based on the detection of two minor variants in *rpoB* (S431T at 33%, and D435V at 9%) that co-occurred with one or more minor variants in other genes (Deeplex Myc-TB targets as well as other genes) sequenced by WGS. None of these minor variants were detected in the two corresponding sputa analyzed by Deeplex Myc-TB (before or after filtering of the data) despite high coverage depths at these positions, suggesting genotypic heterogeneity, or contamination introduced or amplified during subsequent culturing or WGS processing.

Conversely, 23 of the 813 (2.8%) phenotypes predicted as susceptible by PhyResSE were identified as resistant by Deeplex Myc-TB (Supplementary Table S12). Of these, 11 (47.8%) were due to fixed variants or indels detected but not regarded as associated with resistance by PhyResSE. Four mutations (2 T160A, 2 K96N) and 6 frameshift-causing indels or large deletions in *pncA* were found in samples predicted to be MDR with additional resistance to at least ethambutol, in five out of six cases, thus supporting probable pan-first-line resistance (including pyrazinamide). Likewise, one *ethA* frameshift-causing indel was mechanistically [20] predicted to cause resistance by Deeplex Myc-TB and not by PhyResSE. Finally, 12 (52.2%) discordances were due to minority resistance-associated variants at 3.3-12.8%, detected by Deeplex Myc-TB but missed by WGS due to lower read depths (*katG* S315T (n=2); *embB* G406A and co-occurring minor G406A + P404S; *pncA* D8G, H57D, H71Y (n=2) and 1 frameshift-causing insertion; *gyrA* D94A, and *rrs* A514C (n=2)).

The overall proportion of predicted resistance by Deeplex Myc-TB relatively to Mykrobe was somewhat negatively skewed by the individual sensitivity of 58.8% for pyrazinamide, in contrast to 96.6%-100% for all other applicable drugs (Supplementary Table S13). This lower score resulted from the local prevalence of “*M. canettii*” isolates in Djibouti, which are naturally resistant to pyrazinamide and highly restricted to the Horn of Africa [31–34]. While both analysis tools identified “*M. canettii*” in 7 samples based on a fixed synonymous phylogenetic SNP *pncA* A46A, Deeplex Myc-TB did not explicitly report a pyrazinamide resistance prediction on this basis, resulting in 10 pyrazinamide resistant phenotypes predicted by Deeplex Myc-TB versus 17 by Mykrobe. Other discordances resulted from a rare apparent non-sense *rpoB* D435X mutation in a sample and two rare *katG* indels, which were not

reported as rifampicin or isoniazid resistance associated by Deeplex Myc-TB at the time of the study.

Finally, 31 of the 705 (4.4%) susceptible phenotypes predicted by Mykrobe were identified as resistant by Deeplex Myc-TB (Supplementary Table S13). These include the 10 out of 12 phenotypes predicted based on resistance-associated minority variants with frequencies of 3.3-12.8%, and the 7 capreomycin resistant phenotypes due to the fixed *tlyA* N236K mutation. Likewise, 3 of the 5 indels or large deletions in *pncA* ignored by PhyResSE were also unaccounted-for pyrazinamide resistance by Mykrobe. The remaining discordant phenotypes were all due to (near-)fixed variants. These include 7 various mutations in *pncA* (of which 6 were found in samples predicted to be MDR with additional resistance to ethambutol at least, arguing for first-line pan-resistance), a D435A *rpoB* mutation with indeterminate rifampicin association with resistance according to ReSeqTB [7], as well as S297A and Y319S *embB* mutations [35,36] and a *rrs* A906G mutation that were also predicted as resistance-conferring by PhyResSE.

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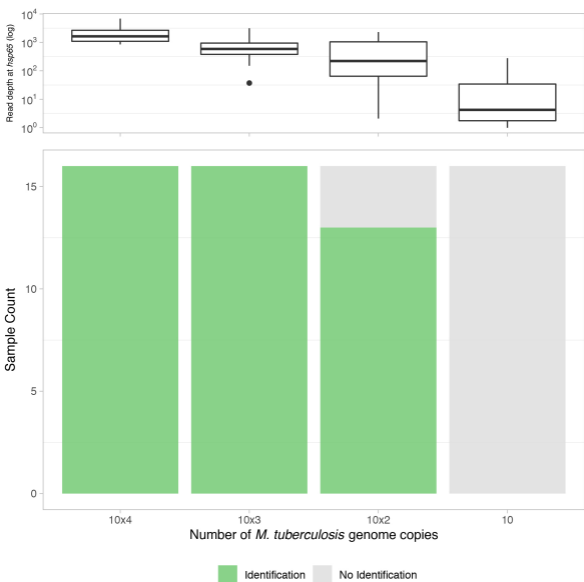
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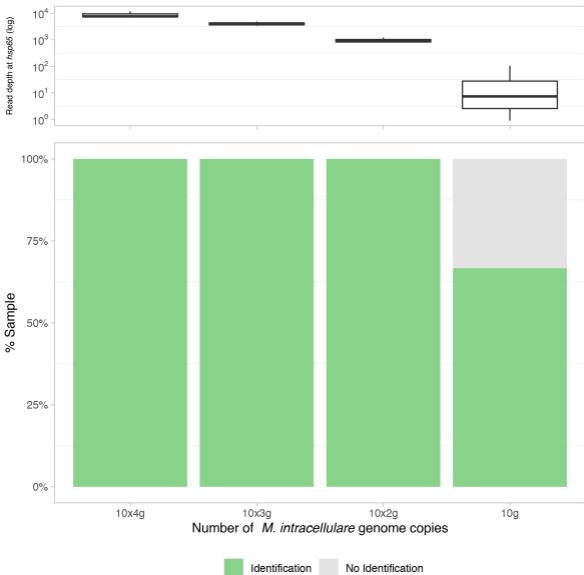
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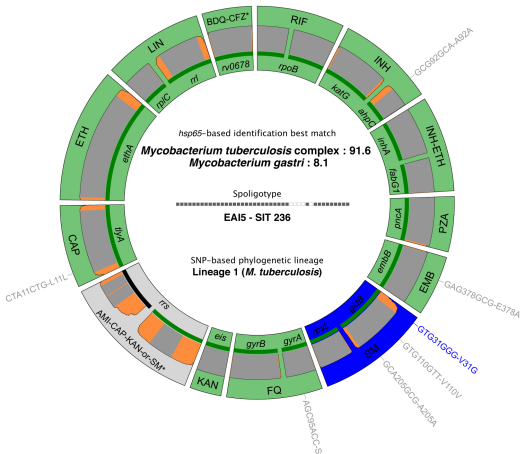
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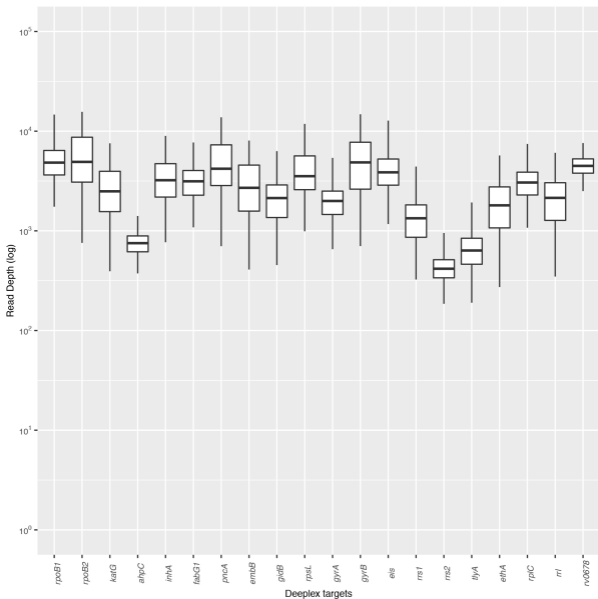
Supplementary Figure 1. Limit of detection (LOD) of Deeplex Myc-TB for mycobacterial identification. (Top) Read depth at *hsp65* versus the number of input genomes. Median values as well as 25-75% quartiles are shown. (Bottom) For each dilution level with 10, 10², 10³, 10⁴ genome copies, LOD was measured as the fraction with (green) or without (grey) correct identification among 16 tests, corresponding to four independent amplification replicates and sequencing rounds of 4 MTBC genomic DNA extracts.



Supplementary Figure S2. Limit of detection (LOD) of Deeplex Myc-TB for NTM identification. (Top) Read depth at hsp65 versus the number of input genomes of a *M. intracellulare* strain. Median values as well as 25-75% quartiles are shown. (Bottom) LOD was measured as the fraction with (green) or without (grey) correct identification in three independent replicated analyses of 10^2 , 10^3 , 10^4 *M. intracellulare* genome copies.



Supplementary Figure S4. Deeplex Myc-TB detecting co-existing *hsp65* reads of *Mycobacterium tuberculosis* complex and *Mycobacterium gastri* strains in DNA from an individual sputum sample collected in a TB drug resistance survey conducted in Djibouti. The respective percentages of *hsp65* reads detected for each species are shown under “hsp65-based identification best-match”. Explanations in the legend of Figure 2 in the main text apply for other parts of this figure.



Supplementary Figure S5. Log read depth obtained at drug resistance-associated Deeplex Myc-TB targets in 429 DNA strain samples from the TDR and the Sciensano-Belgian National TB Reference Center collections.

Median values as well as 25-75% quartiles are shown.

