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Original Article

Concordance of targeted and whole genome sequencing for *Mycobacterium tuberculosis* genotypic drug susceptibility testing

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ABSTRACT

Targeted Next Generation Sequencing (tNGS) and Whole Genome Sequencing (WGS) are increasingly used for genotypic drug susceptibility testing (gDST) of *Mycobacterium tuberculosis*. Thirty-two multi-drugs resistant and 40 drug susceptible isolates from Madagascar were tested with Deeplex® Myc-TB and WGS using the Mykrobe analysis pipeline. Sixty-four of 72 (89 %) yielded concordant categorical gDST results for drugs tested by both assays. Mykrobe didn't detect *pncA* K96T, *pncA* Q141P, *pncA* H51P, *pncA* H82R, *rrs* C517T and *rpsL* K43R mutations, which were identified as minority variants in corresponding isolates by tNGS. One discrepancy (*rrs* C517T) was associated with insufficient sequencing depth on WGS. Deeplex® Myc-TB didn't detect *inhA* G-154A which isn't covered by the assay's amplification targets. Despite those targets being included in the Deeplex® Myc-TB assay, a *pncA* T47A and a deletion in *gid* were not identified in one isolate respectively. The evaluated WGS and tNGS gDST assays show high but imperfect concordance.

1. Introduction

Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant TB (XDR-TB) represent a public health emergency and compromise the achievement of the WHO "End TB" target of disease elimination by 2035 [11]. WHO calls for universal access to drug susceptibility testing (DST) to appropriately guide treatment for all diagnosed TB patients [10]. Whole genome (WGS) sequencing-based or targeted next generation (tNGS) sequencing-based genotypic DST (gDST) assays are available, and their performance was previously evaluated [2,3]. The ability of genotypic assays to detect drug resistance conferring mutations depends on the genomic regions they target, the obtained coverage depths of sequence reads and the mutation catalogue they use as reference [9]. In this study, we compare tNGS using the Deeplex® Myc-TB assay (Genoscreen, Lille, France) and WGS with the Mykrobe analysis pipeline, in their ability to detect drug resistance mutations in a panel of MDR-TB and drug susceptible TB (DS-TB) clinical isolates from the Madagascar national drug resistance surveillance program.

2. Methods

2.1. Study samples

The Madagascar MDR-TB surveillance program performs DST for all patients at higher risk of MDR-TB infection including MDR-TB contacts

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and patients experiencing infection relapse or treatment failure [4,5]. MDR-TB was defined as resistant to at least both isoniazid and rifampicin. Within the surveillance program, isolates were tested using a combination of solid culture-based phenotypic DST (pDST), Xpert MTB/RIF and HAIN line probe assay. When gold standard pDST results were not available, molecular assays were used to determine the MDR profile. All MDR-TB isolates from 2012 to 2017 were included and were matched with DS-TB control isolates in a 1:1 ratio. Matching was based on the region and date of sampling. Isolates were required to pass quality control on both gDST assays for inclusion in the final analysis. In the tNGS analysis, nontuberculous mycobacteria (NTMs) and isolates without identification to the species level were excluded. For WGS (Mykrobe), a TB lineage needs to be identified and the average coverage needs to be 30X or above for isolates to be included. Following this quality control, 32 MDR TB and 40 DS-TB were included in this study.

2.2. Targeted and whole genome sequencing

DNA was extracted from pure mycobacterial culture on Lowenstein-Jensen media using the Van Embden method and was used for gDST methods [7]. Targeted NGS with the Deeplex® Myc-TB assay was performed at Genoscreen (Lille, France) using the manufacturer's amplification kit and Illumina-based amplicon sequencing. The Deeplex® Myc-TB v2.2 pipeline was used for the analysis [3]. This pipeline performs identification of mycobacterial species based on *hsp65* sequencing, TB strain type based on spoligotyping and *M. tuberculosis* complex phylogenetic lineage based on phylogenetic single nucleotide polymorphisms (SNPs), and detects either (i) absence of mutations, (ii) mutations not associated with resistance, (iii) resistance conferring mutations or (iv) mutations of unknown significance in resistance-associated genes. The assay panel covers all first-line (rifampin, isoniazid, ethambutol and pyrazinamide), second-line (quinolones, streptomycin, amikacin, capreomycin, kanamycin, ethionamide) and new or repurposed drugs (linezolid, bedaquiline, clofazimine). As part of the quality control process, isolates not identified as *M. tuberculosis* complex were not included in the analysis. For included samples, when read coverage depth is below a minimum of 5x on a resistance-associated position catalogued in a specific gene target, no prediction is made for the given specific drug.

For WGS, sequencing was performed on a HiSeq2500 illumina platform at the Wellcome Center for Human Genetics (Oxford, UK) and the Mykrobe v0.12.1 pipeline was used for the analysis [1,2]. Mykrobe performs mycobacterial species identification, *M. tuberculosis* complex lineage typing and provides gDST for all first-line dugs, quinolones, aminoglycosides, ethionamide and linezolid using the WHO resistance mutations catalog. Failure to identify a TB lineage or detection of mixed lineages leads to isolates exclusion as part of the quality control process. Minor resistance alleles are not detected by Mykrobe via a hard threshold, as this would inevitably cause problems in high or low coverage samples. Instead, when a low frequency resistance allele is observed, the likelihood of seeing that depth conditional on the genome-wide depth is calculated, and compared with the likelihood under a model where it is caused by sequencing error. For example, 3x depth on a resistance allele is likely to be sequencing error in a sample with 3000x depth, but likely to be a genuine minor resistance allele in a sample with 30x depth.

3. Results

Mykrobe provides a susceptible profile by default when coverage is insufficient. A full WGS-based genotypic profile was hence available for all included isolates. In this context, sequencing depth inferior to 20 % of the expected depth for the sample was considered insufficient. For Deeplex®, seven isolates had incomplete resistotypes and did not yield a prediction for one to five drugs due to insufficient coverage (see Supplementary Material 1). Failure to predict a resistotype occurred for linezolid (n=7), aminoglycosides (n=2) and ethambutol (n=1). Relatively lower rates of amplification of the *rrl* gene target by the Deeplex MyTB assay was previously reported [3]. By design, Mykrobe yielded a prediction for all those drug-isolates combination. This WGS-based prediction was based on sufficient coverage for all but three targets in two distinct isolates. This led to default susceptible predictions despite insufficient coverage for linezolid (n=1) and aminoglycosides (n=1) and ethambutol (n=1).

Concordance between gDST methods was only assessed for drugs for which both genotypic assays provided a prediction. Aggregated results of gDST and pDST profiles are presented in Table 1. Complete DST results for all isolates are presented as Supplementary Material 1. Of all isolates, 8/72 (11 %) yielded discrepant gDST results for at least one drug. One isolate (1 %) yielded discrepant results for 2 distinct drugs (isoniazid and ethionamide) due to a unique mutation, associated with cross-resistance to both drugs, which was only detected by the WGS approach. For first-line drugs, agreement ranged from 94 % (pyrazinamide) to 100 % (rifampicin, ethambutol). For second-line drugs, it ranged from 94 % (streptomycin) to 100 % (quinolones, kanamycin, amikacin, capreomycin and linezolid). Drugs-isolates combinations for which tNGS failed to predict a phenotype or identified a mutation of unknown significance were not considered for the concordance analysis.

Resistance conferring mutations from discordant isolates are presented in Table 2. All Mykrobe WGS and Deeplex tNGS resistance mutations and sequencing metrics are presented in supplementary materials 2. Three mutations were exclusively detected using Mykrobe: *inhA* G-154A (alias *fabG1* L203L) (isoniazid and ethionamide), *pncA* T47A (pyrazinamide) and deletion gid_GGCCGTTGAGATCGTGCGGGGGCG330TGCCGTTGAGATCGTGCGGGGG (streptomycin). The *inhA* G-154A mutation is situated in a genomic region which is not amplified in the current Deeplex® Myc-TB assay design. For the two other targets, Deeplex® Myc-TB reported those isolates as wild type and susceptible based on sufficient coverage. For those specific drug-isolates discrepancies, pDST was either unavailable (n=2, *pncA* T47A for pyrazinamide and *inhA* G-154A for ethionamide), resistant (n=1, *inhA* G-154A for isoniazid) or susceptible (n=1, *gid* deletion). In six isolate-mutation combinations, resistance were exclusively predicted by Deeplex®: *pncA* K96T, *pncA* Q141P, *pncA* H51P, and *pncA* H82R (pyrazinamide), *rrs* C517T and *rpsL* K43R (streptomycin). These mutations were all minority variants, detected with very low frequencies - from 2.7 % (*pncA* H51P) to 14.7 % (*rpsL* K43R), with the relative exception of *pncA* Q141P (detected at 39.9 % of read frequency). All those mutations are included in the Mykrobe v0.12.1 and the WHO catalogues. As a reflection, two of those missed mutations were accurately identified in other isolates: *pncA* Q141P in 2-42 and *rpsL* K43R in 1-5 and 135. These mutations were detected at 47.7 % and above 90 % frequencies in the respective isolates by Deeplex Myc-TB. For those specific drug-isolates discrepancies, pDST was either unavailable (n=4, *pncA* K96T, *pncA* Q141P, *pncA* H51P, and *pncA* H82R for pyrazinamide) or susceptible (n=2, *rrs* C517T and *rpsL* K43R for streptomycin).

4. Discussion

This study compared gDST predictions from WGS (*Mykrobe)* and tNGS (Deeplex® Myc-TB) in a collection of MDR-TB and DS-TB clinical isolates. This study sampling strategy allowed to capture a representative sampling of the circulating TB drug resistance mutations in the country and identified a fair number of discrepancies between gDST assays' results. There were 8/72 (11 %) isolates exhibiting discrepant categorical results for at least one drug and total agreement between methods was above 94 % for all first and second-line drugs. Although other commercial tNGS assays (NanoTB®, TBseq®) and other open access TB WGS analysis pipelines are available, the goal of this study was to confirm the impact of the sequencing approach and compare results of both end-to-end assays. We did not perform benchmarking of bioinformatic tools which has already been performed [8]. Given the

Table 1
Concordance between genotypic and phenotypic drug susceptibility testing

Drugs	Phenotype S						Phenotype R						Phenotype Unknown						Concordance
	RR	SS	RS	SR	RU/F	SU/F	RR	SS	RS	SR	RU/F	SU/F	RR	SS	RS	SR	RU/F	SU/F	% (95 %-CI)
rifampicin	0	38	0	0	0	1	31	2	0	0	0	0	0	0	0	0	0	0	100 % (94.9 – 100.0)
isoniazid	1	32	0	0	0	5	28	2	1	0	1	2	0	0	0	0	0	0	98 % (91.6 – 100.0)
pyrazinamide	0	0	0	0	0	0	0	0	0	0	0	0	7	60	1	3	0	1	94 % (86.2 – 98.4)
ethambutol	11	47	0	0	0	2	9	2	0	0	0	1	0	0	0	0	0	0	100 % (94.8 – 100.0)
streptomycin	7	34	1	2	2	19	4	1	0	0	0	0	0	2	0	0	0	0	94 % (83.8 – 98.8)
quinolones	0	34	0	0	0	2	0	0	0	0	0	0	0	35	0	0	0	1	100 % (94.8 – 100.0)
amikacin	1	29	0	0	0	6	0	0	0	0	0	0	0	28	0	0	0	8	100 % (93.8 – 100.0)
capreomycin	1	28	0	0	0	6	0	1	0	0	0	0	0	29	0	0	0	7	100 % (93.9 – 100.0)
kanamycin	1	28	0	0	0	7	0	0	0	0	0	0	0	29	0	0	0	7	100 % (93.8 – 100.0)
ethionamide	0	0	0	0	0	0	0	0	0	0	0	0	7	60	1	0	0	4	99 % (92.1 – 100.0)
linezolid	0	0	0	0	0	0	0	0	0	0	0	0	0	65	0	0	0	7	100 % (94.5 – 100.0)

F; Failed, R; resistant S; susceptible, U; unknown. For each column, the first and second letters respectively represent Mykrobe WGS and Deeplex Myc-TB tNGS resistance prediction.

Table 2
Resistance conferring mutations in isolates exhibiting genotypic testing categorical discrepencies

Sample ID	Drug	Discordances						Molecular basis for resistance (% variant frequency by WGS or tNGS)	pDST	Catalogue/target inclusion		
		WGS Mykrobe			tNGS Deeplex®-Myc-TB							
		Prediction	Sequencing depth (ref/alt)	Lineage	Prediction	Sequencing depth (mean depth/coverage breath)	Lineage		LJ proportion	WHO	Mykrobe	Deeplex®-Myc-TB
105	Isoniazid-ethionamide	R	0/43	4.1.2.1	S	ND	Other than 4.9[1]	inhA_G-154A[2] (or fabG1_L203L) (100%)	R	Yes	Yes	No[3]
1-1		R	0/61	1.1.2	S	4880.63 / 100	"Mixed lineage 4.6.2 (23%) 1.1.2 (66%)"	pncA_T47A (100%)	U	Yes	Yes	Yes
1-3	pyrazinamide	S	66/1	1.1.2	R	7815.74 / 100.0	1.1.2	pncA_K96T (5.0%)	U	Yes	Yes	Yes
1-14		S	42/1	1.1.2	R	5461.02 / 100.0	1.1.2	pncA_Q141P (39.9%)	U	Yes	Yes	Yes
1-14		S	50/0	1.1.2	R	5461.02 / 100.0	1.1.2	pncA_H51P (2.7%)	U	Yes	Yes	Yes
1-46		S	41//3	1.1.2	R	428.75 / 100.0	1.1.2	pncA_H82R (3.4%)	U	Yes	Yes	Yes
1-33	streptomycin	R	55/55	1.1.2	S	1549.31 / 100.0	1.1.2	gid_GGCCGTTG AGATCGTGCG GGGGCG330TG CCGTTGAGATC GTGCGGGGG (50%)	S	Yes	Yes	Yes
1-38		S	9/2	4.1	R	109.63 / 99.11	Other than 4.9[1]	rrs_C517T (5.8%)	S	Yes	Yes	Yes
2-46		S	56/1	1.1.2	R	4791.23 / 100.0	1.1.2	rpsL_K43R (14.7%)	S	Yes	Yes	Yes

LJ; Lowenstein Jensen, R; resistant S; susceptible, U; unknown; ND, not detected.
[1] Other than lineage 4.9 (that of H37Rv) is called for isolates with a SNPs profile suggesting sublineage 4 but not that of H37Rv.
[2] *inhA* G-154A and *fabG1* L203L are the same mutation.
[3] *inhA* G-154A (alias *fabG1* L203L) is not included in the genomic regions amplified by the current Deeplex Myc-TB assay.

recent curation and publication of a WHO endorsed geno-to-pheno mutation catalog, this component of gDST assays' performance is expected to become more and more standardized across available pipelines [12].

Our analysis allowed us to categorize observed discrepancies as resulting from either i) minority resistance variants missed by insufficient sequencing coverage depth/underperforming limit of detection of mutant subpopulations by WGS and ii) one out-of-target mutation in a gene region that is currently not covered by the tNGS assay. Interestingly, most of the minority variants missed by WGS but detected by deeper sequencing with Deeplex® Myc-TB were pyrazinamide resistance-associated mutations (*pncA* K96T, *pncA* Q141P, *pncA* H51P, and *pncA* H82R) that were all found in MDR-TB genotypic profiles. Such recurrent association with MDR supports the authenticity of these low frequency variants detected by tNGS-based deep sequencing, as the pretest probability of pyrazinamide resistance associated with non-synonymous *pncA* mutations is high in an isolate that is resistant to rifampicin. However, this conclusion could not be independently assessed by phenotypic testing, as phenotyping was not available for isolates exhibiting discrepant gDST results for pyrazinamide. The tNGS increased efficiency at identifying minor mutated subpopulations was previously described [3]. For two isolates where Deeplex® Myc-TB detected minority streptomycin resistant variants that were below detection level by WGS, LJ-based pDST found those to be drug-susceptible. The *inhA* G-154A mutation is not included in the amplified *inhA* region in the current version of Deeplex Myc-TB, which led to one isoniazid gDST discrepant profile in our study. In one other isolate, Mykrobe WGS predicted drug resistance to streptomycin based on detected deletion in *gidB*. In contrast, wild type *gidB* sequence was detected at a mean read depth of 1549X by Deeplex® Myc-TB and this isolate was also found to be susceptible on pDST. Mutations of unknown significance were not considered in the concordance analysis, which represents a limitation, as publicly available and analysis pipeline proprietary databases do not always provide the same interpretation for the same mutation.

We considered the possibility of laboratory errors or culture isolates and DNA extract mishandling to account for some of the observed discordances between gDST assays. Only 1 out of 8 isolates exhibited discrepant results involving more than one resistance mutations, which however concerned a same target (i.e. Two different *pncA* mutations associated with pyrazinamide resistance detected as minority variants by tNGS in the MDR isolate 1-14). No pattern of systematic disagreement between one or both gDST assays and phenotypic testing was observed. For all but one isolates exhibiting discrepant profiles, SNP-based MTB lineage typing were identical or compatible (in case of "Other than H37Rv", indicating a lineage other than 4.9 by deeplex Myc-TB) on both assays. For one isolate, tNGS detected the presence of mixed lineages (23 % 4.6.2 and 66 % 1.1.2) while WGS only identified the dominant lineage. We are hence not able to suggest or confirm isolates or DNA extract mishandling.

WGS has the relative advantage of enabling the identification of "out of targets" or new candidate resistance mutations when facing pDST/tNGS discrepancies or pDST resistance to new or repurposed drugs for which resistance catalogues are still being developed. This occurred only once in our study with *inhA* G-154A (alias *fabG1* L203L). WGS also has the advantage to enable higher resolution molecular typing for surveillance and transmission investigations. This could not be assessed as part of this study given the absence of suspected epidemiological links between patients. Targeted sequencing involves PCR target amplification and can thus be performed directly from clinical sputum samples. This is a significant advantage over the WGS approach which requires fastidious and lengthily culture amplification to generate sufficient DNA template for sequencing. Moreover, as also seen here, the substantially greater sequencing depths that can be obtained by tNGS allow more sensitive detection of resistance mutations borne by minority populations, which are not always identified by pDST but can be predictive of treatment failure [6]. In our study, both gDST methods were performed on DNA extracts from pure culture. Therefore, we could not measure the potential impact of working directly from sputum on the analytical sensitivity of the targeted assay. Another limitation of our study is that according to the Mykrobe analysis pipeline quality control features, isolates exhibiting mixed lineages were excluded what may have led us to underestimate the number of gDST discrepant isolates.

Discrepancies between different sequencing platforms and analysis tools will remain despite standardization of back-end mutation catalogs. For low-income and TB high-burden countries alike Madagascar, many factors beyond the analytical performance will have to be considered when deciding to include a gDST assays in their diagnostic algorithm. Those include (i) costs, both as the required up-front capital investment and the per test expenses, (ii) usability of bio-informatics pipeline solutions in settings with limited programming capacity, and (iii) desired integration with other diagnostics tests (phenotyping) and sample workflow (tNGS on sputum vs WGS on pure culture). Laboratories implementing gDST for the first time should also consider whether the ability of providing WGS-based high resolution molecular epidemiology data to public health authorities has added value in their setting.

Albeit largely concordant, WGS (Mykrobe) and tNGS (Deeplex® Myc-TB) assays have intrinsic design characteristics which may lead to discrepant gDST predictions in a relatively small proportion of isolates. Improved sequencing protocols as well as further update of resistance-associated amplification targets and reference mutation catalogues are mandatory to maintain the high clinical performance of both assays [12].

Ethics and consent

This study was performed on anonymized retrospective isolates and patients' informed consent was hence not required as per local ethics board.

CRediT authorship contribution statement

William Cloutier Charette: Conceptualization, Data curation, Formal analysis, Visualization, Writing – original draft. **Marie-Sylvianne Rabodoarivelo:** Conceptualization, Data curation, Investigation. **Floriane Point:** Data curation. **Astrid M. Knoblauch:** Data curation, Supervision, Validation. **Fanantenana Randria Andrianomanana:** Data curation, Investigation. **Michael B. Hall:** Data curation, Investigation, Methodology, Software, Supervision, Validation. **Zamin Iqbal:** Conceptualization, Investigation, Methodology, Software, Validation. **Philip Supply:** Data curation, Investigation, Methodology, Software, Validation, Visualization. **Anandi Martin:** Conceptualization, Funding acquisition, Methodology, Resources. **Niaina Rakotosamimanana:** Conceptualization, Methodology, Project administration, Visualization. **Simon Grandjean Lapierre:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Philip Supply reports a relationship with GenoScreen that includes: consulting or advisory. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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