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Early View

Research letter

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Rapid genomic first- and second-line drug resistance prediction from clinical *Mycobacterium tuberculosis* specimens using Deeplex®-MycTB

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Short Abstract

Targeted next generation sequencing using the Deeplex-MycTB assay can rapidly generate comprehensive drug resistance profiles from *Mycobacterium tuberculosis* complex cultures and directly from tuberculosis patient samples to guide personalized treatment.

To the Editor:

The emergence of multidrug-resistant (MDR) and extensively-drug-resistant (XDR-TB) *Mycobacterium tuberculosis* complex (Mtbc) isolates challenges tuberculosis (TB) control worldwide [1] and requires the rapid determination of extensive resistance profiles [2], enabling prompt initiation of effective treatment regimens. Phenotypic drug susceptibility testing (pDST) takes up to six weeks [3], and is unreliable and/or not standardized for several drugs according to WHO guidelines [4]. Molecular DST (mDST) assays, like Xpert MTB/RIF (Cepheid) or MTBDR*plus*/*sl* line probe assays (LPAs, Hain Lifesciences) can be performed directly from clinical specimens, but only target a limited number of resistance variants [5].

This limitation can be overcome by whole-genome sequencing (WGS) allowing for the most comprehensive analysis for resistance variants [6]. However, direct sequencing of sputum samples is challenging due to low amounts of Mtbc DNA [7].

To overcome this difficulty, an all-in-one targeted deep-sequencing assay of a 24-plexed amplicon mix, named Deeplex®-MycTB (Genoscreen) has been developed. The assay covers 18 regions associated with drug resistance (*rpoB, katG, fabG1, ahpC, inhA, pncA, embB, gidB, rpsL, gyrA, gyrB, ethA, eis, rrs, tlyA, rplC, rrl, rv0678*), and enables species identification and genotyping of Mtbc isolates. However, very limited data are available on the performance of this assay for predicting resistance [5,8]. Therefore, we evaluated the performance of the Deeplex®-MycTB assay for resistance and susceptibility prediction from DNA extracts from 81 cultured isolates and 50 clinical samples, by comparison with results from WGS, pDST and/or MTBDR*plus*/*sl* LPAs.

DNA was obtained from cultured isolates from an Mtbc isolate collection from Sierra Leone [9]. Clinical specimens were collected at the German National Reference Laboratory for Mycobacteria from January-November 2018 by convenience sampling, stored at -80°C and processed as follows: after decontamination/homogenization (2-5 ml sample) with 3% NaOH/NALC, heat inactivation (20 min at 95°C), DNA was purified by using the QIAamp DNA-Mini-Kit (Qiagen).

0.9 ng of DNA from cultured isolates or 9 µl of DNA extract from clinical samples were used for Deeplex®-MycTB PCR. Next-generation sequencing (NGS) was performed using 1 ng of prepared amplicons as input for the Nextera XT library preparation kit (Illumina) and sequencing on the MiniSeq and NextSeq 500 platforms. FASTQ files were uploaded to the cloud-based analysis software (app.deeplex.fr) or analysed with the MTBseq pipeline when performing WGS of genomic DNA from cultured isolates [10].

Using the Deeplex®-MycTB assay, an average read coverage depth between 2,617 and 7,593 was obtained on individual samples with a mean average coverage depth of 4,476 over the reference targets across the 81 samples. On DNA samples from Sierra Leone [9], 466 variants were detected by Deeplex®-MycTB in the 18 resistance genes analysed, while WGS analysis identified 464 of these 466 variants. Of these, 100 and 98 were annotated as resistance mediating mutations by Deeplex®-MycTB and WGS, respectively. Accordingly, the overall concordance between both assays was 99.6% for all SNPs with 98% for resistance mutations. The discordance was 2 minority resistance variants (frequency below 50%) in *rpoB* and *embB* with a frequency of 6.8% and 4.9%, determined by the Deeplex®-MycTB assay. These mutations were detected by Deeplex®-MycTB with amplicons at 2,222-fold and 11,901-fold coverage depths, respectively, but were undetected by WGS due to comparatively lower coverage depths on the corresponding gene positions.

When testing the performance of Deeplex®-MycTB on 50 clinical samples, complete resistance predictions with Deeplex®-MycTB could be made for 39. All smear positive (+, 2+, 3+) samples resulted in a sequencing acceptability grading of +, 2+ or 3+, by the Deeplex®-MycTB software, and allowed complete resistance predictions. In addition, five out of seven (71.4%) smear +/- samples and two out of nine (22.2.%) smear negative samples also generated fully interpretable sequencing results. Overall, seven out of eleven samples that failed were microscopically negative, two were +/-, and for two samples no microscopy results were available. Overall, this resulted in the detection of 45 resistance mediating variants in *rpoB, katG, inhA, pncA, embB, gidB, rpsL, gyrA, eis, rrs,* and *ethA*. Of those, 26 variants were fixed variants (frequency of at least 97%), five were unfixed variants

(frequencies below 97%) in genes *rpoB*, *pncA*, *embB*, *rrs* and *ethA* and 14 were minority variants (below 50%) in genes *rpoB*, *katG*, *ahpC*, *pncA*, *embB*, *gidB*, *rrs*, *gyrA* (n=5) and *ethA* (n=2).

All of the 16 fixed or majority resistance-mediating mutations (frequency of at least 97%), detected by Deeplex®-MycTB in the regions covered by MTBDRplus/*sl* LPAs were concordantly detected by LPA testing, also performed on clinical samples (Tab.1). However, three instances of minority variants in *rpoB* (S450L; 27.8%), *katG* (S315G; 5.5%) and *gyrA* (A90V, 10.5%, 19.3%, 25.9%, 28%) detected by Deeplex-MycTB were undetected by LPAs also in repeated assays. This is somewhat surprising as previous papers suggest a lower mixed population threshold of ≥5% for LPAs [11]. However, this may be variable and depend e.g. on the nature of the sample and the binding kinetics of particular probes.

Concordances between predictions of resistance and susceptibility by Deeplex®-MycTB and pDST data for the first-line drugs obtained after culture of these clinical samples ranged between 94.9% (INH) and 97.4% (RIF, PZA, EMB) (Tab.1). For second-line drugs, the concordances ranged from 66.7% (FQ) to 75% (PTH) and 100% (aminoglycosides, LIN, BDQ).

The discordance was observed for samples with minority variants known to confer low-level resistance. One sample contained Mtbc DNA with a *katG* S315G mutation reportedly associated with low-level resistance to INH [12] at a frequency of 5.5%. The mutation was not detected by LPA and the culture isolate subsequently tested susceptible at the INH critical concentration (CC). Four samples contained minority *gyrA* A90V subpopulations reported to confer low-level resistance to MXF [13], which are particularly difficult to detect phenotypically when present at low frequency only. Likewise, these subpopulations were not detected by LPAs and showed susceptible phenotypes at the MXF CC. The single discordance among 39 samples phenotypically tested for PZA carried a *pncA* Q10P mutation at a frequency of 10.6%. Moreover, pDST for PZA is considered less reliable [14]. For RIF, the single discordance consisted of a sample carrying a low-level resistance *rpoB* H445D (99.7 %) mutation, which tested RIF susceptible by pDST at the CC. This mutation is

often linked with susceptible pDST results but is known to confer clinical resistance to RIF [15].

The remaining discordance included a sample without detected INH resistance-associated mutation that tested INH resistant by pDST, two samples without ETH/PTH resistance-associated mutations tested PTH resistant by pDST, and one sample carrying an *embB* G406S mutation tested EMB susceptible by pDST (Tab.1). The *embB* G406S mutation is associated with resistance to EMB. However, the pDST is known to be subjected to reproducibility problems [16].

The analytical range of Deeplex®-MycTB is superior to other mDST assays due to Deeplex®-MycTB covering resistance-mediating genes for all WHO group A and B drugs and covering much larger regions (*rpoB*, *katG*, *embB*, *rrs*). This advantage has been illustrated in a study performed in South Africa, reporting MDR outbreak isolates carrying a I491F mutation outside the RIF resistance-determining region [5].

Comprehensive interrogation of genomic regions involved in resistance development is crucial for the prediction of susceptibility and resistance, which is potentially allowing to replace pDST for a large fraction of diagnostic samples. Here, all of the 98 resistance-associated mutations detected by WGS were also identified by Deeplex®-MycTB in the tested isolates. However, data included mainly mutations conferring resistance to first-line drugs.

Further, another low-level frequency variant (18.4%) detected in *ahpC* (G-48A) by Deeplex®-MycTB and missed by the LPA in a sample, was confirmed as INH resistant by pDST, thus supporting the superiority of Deeplex®-MycTB to detect heteroresistance.

In conclusion, our results suggest that the Deeplex®-MycTB assay is a valuable tool for rapid resistance and drug susceptibility testing in TB diagnostics, with the potential to replace pDST for a large fraction of patients with drug susceptible, MDR, and XDR-TB. Especially when performed on clinical specimens, this test will reduce the load of pDST in TB diagnostic laboratories and the turnaround time for reporting. The more comprehensive coverage of resistance targets, and its capability to detect bacterial subpopulations, are

important advantages compared to other mDST assays [17]. Additional prospective studies with larger sample numbers are under way to further assess the challenges in implementation and performance characteristics of Deeplex®-MycTB in settings with different epidemiological backgrounds and infrastructure.

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Tab. 1: Prediction of resistance and susceptibility by the Deeplex®-MycTB assay for 39 clinical samples compared to phenotypic DST results and Hain MTBDR*plus and* MTBDR*sl* assay results.

		Deeplex®-MycTB assay			**pDST**			**Hain MTBDR*plus/sl***		
drug	**locus**	**R**	**R (minority mutation)**	**S**	**R**	**S**	**concordance**	**R**	**S**	**concordance**
RIF	*rpoB*	8	0	31	7[1]	32[1]	38/39[1] (97.4%)	8[10]	31	39/39 (100%)
INH	*katG/fabG1/ ahpC/inhA*	6	2	31	8[2]	31[2]	37/39[2] (94.9%)	4[11] 3	34 34	37/38 (97.4%) (*katG*) 37/37 (100%) (*inhA*)
PZA	*pncA*	4	1	34	4	35[3]	38/39[3] (97.4%)	-	-	-
EMB	*embB*	4	0	35	3[4]	36[4]	38/39[4] (97.4%)	1	2	3/3 (100%)
SM	*gidB/rpsL/rrs*	5	2	32	nd	nd	nd	-	-	-
FQ	*gyrA/gyrB*	0	4	35	0[5]	12[5]	8/12[5] (66.7%)	0[12] 0	39 39	35/39 (89.7%) (*gyrA*) 39/39 (100%) (*gyrB*)
ETH/ PTH	*inhA/fabG1/ inhA*	3	0	36	5[6]	3[6]	6/8[7] (75.0%)	3	34	37/37 (100%)
KAN only	*eis*	1	0	38	0[7]	4[7]	4/4[8] (100%)	-	-	-
KAN AMI CAP	*rrs*	1	0	38	1[8]	7[8]	8/8[8] (100%)	1	38	39/39 (100%)
CAP only	*tlyA*	0	0	39	0	5	5/5[9] (100%)	-	-	-
LIN	*rplC/rrl*	0	0	39	0	2	2/2 (100%)	-	-	-
BDQ CFZ	*Rv0678*	0	0	39	0	2	2/2 (100%)	-	-	-

RIF, rifampicin (critical concentration 1.0 µg/ml); INH, isoniazid (0.1 µg/ml); PZA, pyrazinamide (100 µg/ml); EMB, ethambutol (5 µg/ml); SM, streptomycin; FQ, fluoroquinolones (moxifloxacin 0.25 µg/ml and 1 µg/ml); ETH, ethionamide; PTH, prothionamide (2.5 µg/ml); KAN, kanamycin (2.5 µg/ml); AMI, amikacin (1.0 µg/ml); CAP, capreomycin (2.5 µg/ml); LIN, linezolid (1.0 µg/ml); BDQ, bedaquiline (1.0 µg/ml); CFZ, clofaximine

(1.0 µg/ml); R, number of isolates tested resistant; S, number of isolates tested susceptible; nd, not done; second line phenotypic DST testing was only performed on MDR isolates. pDST was performed in the BACTEC MGIT960 system. From the 11 specimens that failed to give an interpretable sequencing result with the Deeplex®-MycTB assay, 7 were microscopy negative, 2 were +/- and for 2 no microscopy result was available.

[1] A sample carrying a "disputed" rpoB H445D (99.7%) was tested RIF S by pDST [15].

[2] One sample without mutation tested INH R by pDST; one sample with a minority, low level INH resistance-conferring mutation katG S315G (5.5%) was tested INH S by pDST [12].

[3] One sample with a minority mutation pncA Q10P (10.6%) was tested PZA S by pDST.

[4] One sample with a resistance-associated embB G406S mutation (99.7%) was tested EMB S by pDST.

[5] Four samples with minority, low level moxifloxacin-conferring mutation gyrA A90V (10.5%, 19.3%, 25.9%, 28%) tested moxifloxacin S [13]; eight without mutation were concordantly tested FQ S.

[6] Two samples without resistance-associated mutations tested PTH R by pDST; three other samples with such mutations and three without such mutations concordantly tested PTH R and PTH S by pDST, respectively.

[7] Four samples without mutations tested KAN S; the sample with a KAN R-associated mutation was not tested.

[8] One sample with a resistance-associated mutation and seven without such mutations concordantly tested KAN, AMI and/or CAP R and S by pDST, respectively.

[9] Five samples without resistance-associated mutation concordantly tested CAP S.

[1o] *rpoB* S450L (27.8%; in combination with *rpoB* H445D at 75.3% in a same sample) was not detected by Hain MTBDR*plus.*

[11] *katG* S315G (5.5%) was not detected by Hain MTBDR*plus.*

[12] Minority *gyrA* A90V variants (10.5%, 19.3%, 25.9% in combination with *gyrA* D94G minority variant at 8.3% in a same sample, 28%) not detected by Hain MTBDR*sl.*

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