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ANALYTICAL PROCEDURES

Utility of Targeted, Amplicon-Based Deep Sequencing To Detect Resistance to First-Line Tuberculosis Drugs in Botswana

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ABSTRACT Multidrug-resistant tuberculosis (TB) is an alarming threat, and targeted deep sequencing (DS) may be an effective method for rapid identification of drug-resistant profiles, including detection of heteroresistance. We evaluated the sensitivity and specificity of targeted DS versus phenotypic drug susceptibility testing (pDST) among patients starting first-line anti-TB therapy in Botswana. Overall, we found high concordance between DS and pDST. Lower sensitivity of DS, which targets established high-confidence resistance variants, was observed for detecting isoniazid resistance among HIV-infected patients.

KEYWORDS HIV infections, next-generation sequencing, diagnostics, drug-resistant tuberculosis, heteroresistance, single-molecule overlapping reads

The recent rollout of molecular-based diagnostic approaches represents an important step forward in reducing the time required to identify multidrug-resistant tuberculosis (MDR-TB; defined as resistance to both isoniazid [INH] and rifampin [RIF]) (1). However, widely used methods such as GeneXpert MTB/RIF and line-probe assays may not detect drug-resistant strains when they represent a small proportion of the population due to microevolution or concomitant infection with drug-susceptible strains (mixed infections) (2, 3). Targeted deep sequencing with the single-molecule-overlapping read (SMOR) assay is a promising molecular approach that allows rapid characterization of drug-resistant profiles by targeting resistance-conferring mutations at multiple bacterial loci with high levels of sequencing depth (e.g., >10,000× coverage) (4–6) and enables the identification and quantification of rare and ultrarare genetic variants down to a resolution as low as 0.1% in a heterogeneous sample (7).

We examined the clinical utility (sensitivity and specificity) of deep sequencing in detecting INH and RIF resistance at reported analytical sensitivities for detecting minority drug-resistant variants (resistance-associated variants [RAV]) for GeneXpert MTB/RIF (≥50%) (8), line-probe assays (≥5%) (2), and phenotypic drug susceptibility testing (pDST) with the mycobacteria growth indicator tube (MGIT; ≥1%) (2) among patients starting first-line anti-TB therapy in Botswana (1). From 2012 to 2016, the Kopanyo study enrolled all patients diagnosed with TB in the Gaborone and Ghanzi districts of Botswana (9). *Mycobacterium tuberculosis* DNA was extracted from positive sputum cultures of recruited patients and underwent 24-locus mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) genotyping.

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TABLE 1 Characteristics of participants[a]

Characteristic	No. of HIV-negative patients (%) or median (IQR)	No. of HIV-positive patients (%) or median (IQR)	All[b] patients (%) or median (IQR)
District			
Gaborone	79 (73.8)	122 (91.0)	207 (83.5)
Ghanzi	28 (26.2)	12 (9.0)	41 (16.5)
Gender			
Male	63 (58.9)	78 (58.2)	145 (58.5)
Female	44 (41.1)	56 (41.8)	103 (41.5)
Age in years	28.4 (22.0–38.0)	36.7 (32.6–44.0)	34.2 (26.4–42.0)
Prior hospitalization			
No	64 (59.8)	70 (52.2)	139 (56.0)
Yes	24 (22.4)	40 (29.9)	66 (26.6)
Unknown	19 (17.8)	24 (17.9)	43 (17.3)
Prior TB			
No	95 (88.8)	110 (82.1)	211 (85.1)
Yes	12 (11.2)	24 (17.9)	37 (14.9)
Smear microscopy			
Negative	12 (11.2)	31 (23.1)	46 (18.5)
Positive	95 (88.8)	103 (76.9)	202 (81.5)
Phenotypic DST			
Susceptible	102 (95.3)	120 (89.6)	229 (92.3)
INH-monoresistant	2 (1.9)	6 (4.5)	8 (3.2)
RIF-monoresistant	0	4 (3.0)	4 (1.6)
MDR-TB	3 (2.8)	4 (3.0)	7 (2.8)
$CD4^+$ T cell count	NA	196 (104–394)	NA

[a]IQR, interquartile range; DST, drug susceptibility testing; INH, isoniazid; RIF, rifampin; MDR-TB, multidrug-resistant TB; NA, not available.
[b]Includes 7 patients with unknown HIV status.

Based on MIRU-VNTR, 99 patients with mixed or possibly mixed infections (defined as the presence of ≥2 repeats at one or more loci in the same sputum sample) and 200 patients randomly selected from 1,396 patients with single-strain infection were included in the present study. *M. tuberculosis* DNA from primary cultures used for MIRU-VNTR underwent targeted deep sequencing with the SMOR assay (5, 6). We targeted three critical gene regions known to confer resistance with a 25,000× depth of coverage, *inhA* promoter and the *katG* gene (associated with INH resistance) (10) and the RIF resistance-determining region (RRDR) of the *rpoB* gene (11). Fifteen samples with an average depth of <2,000 total reads were excluded to minimize misclassification of low-frequency variants due to sequencing errors (6). For the RIF specificity analysis, an additional 19 samples were excluded due to failed deep sequencing reads. pDST was performed for all cultured isolates with the MGIT 960 system and was used as the reference standard for sensitivity and specificity calculations. All discordant samples between deep sequencing and pDST were retested with MGIT.

Overall, 241 patients with known HIV status and pDST results were included in the final analysis (Table 1). Of these, 222 (92.1%) patients had *M. tuberculosis* strains that were susceptible to both drugs, 8 (3.3%) were monoresistant to INH, 4 (1.7%) were monoresistant to RIF, and 7 (2.9%) were resistant to both drugs (MDR-TB).

Deep sequencing of the targeted loci correctly identified those who were phenotypically susceptible to RIF (211/211; 100% specificity) and to INH (226/226; 100% specificity), regardless of HIV infection status (Fig. 1). Among 11 specimens that showed phenotypic resistance to RIF, deep sequencing identified all 11 as genotypically resistant (11/11; 100%), regardless of HIV infection status. Among HIV-infected patients, targeted deep sequencing detected 6 out of 10 patients with phenotypic INH resistance at RAV frequency of ≥50% and 7 out of 10 patients at RAV frequency of ≥5% and ≥1%

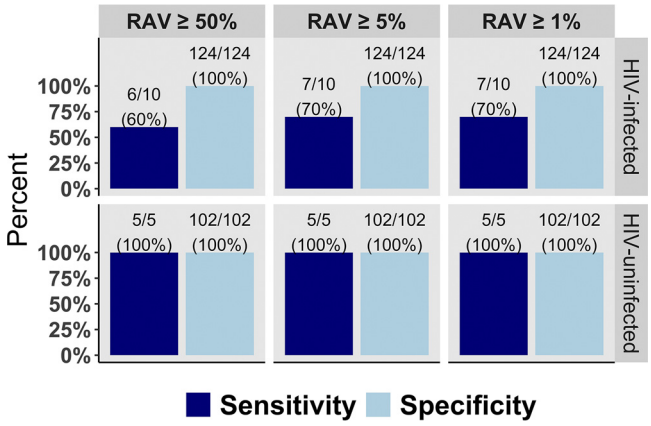

FIG 1 Sensitivity and specificity of targeted deep sequencing in detecting INH resistance at different RAV frequencies, stratified by HIV infection status. INH, isoniazid; RAV, resistance-associated variants.

(versus 100% among HIV-uninfected patients; Fig. 1). The overall sensitivity of deep sequencing for INH resistance detection (regardless of HIV infection status) was 80% (12/15) at RAV frequency of ≥1%. All INH-resistant isolates had a MIC of 0.2 μg/ml. No differences were found between isolates with different RAV frequencies. We verified all discordant samples at RAV frequency of ≥5% via SMOR and confirmed that there were no minor variants or any other single-nucleotide polymorphisms (SNPs) occurring within the *inhA* promoter and *katG* high-confidence resistance regions.

Table 2 shows the characteristics of patients with discordance between deep sequencing and pDST at RAV frequency of ≥50%. All four discordant cases were HIV positive; one had single-strain infection, two had possibly mixed infections, and one had mixed infection with 17% RAV detected in the *katG* gene.

Overall, we found a very high concordance between deep sequencing and pDST among patients starting first-line TB therapy in Botswana. Sensitivity of deep sequencing of the targeted loci may be lower for detecting INH resistance among HIV-infected individuals. Notably, we were able to detect an INH-resistant isolate with 17% RAV in the *katG* gene, which could have been missed by less sensitive tests. This indicates that deep sequencing could, in principle, improve early detection of INH resistance compared to conventional molecular tests in clinical settings where there is a high prevalence of heteroresistant infections.

Our findings are consistent with studies by other groups that observed reduced sensitivity for molecular detection of INH resistance in high-HIV settings. Dorman et al. reported 62% sensitivity for MTBDR*plus* v1 in detecting INH resistance among TB patients in South Africa (12), while Luetkemeyer et al. reported 70.6% sensitivity among HIV-infected patients in South Africa, Botswana, and South America (13). It is possible that the mechanisms of acquiring resistance are different in some HIV-positive patients who were previously treated with INH preventive therapy (14).

Our findings also highlight the possibility that noncanonical mutations not covered

TABLE 2 Characteristics of patients with discordance between deep sequencing and pDST at RAV frequency of ≥50%[a]

Patient	Gender	Age (yr)	HIV	CD4$^+$ T cell count	Smear microscopy	pDST INH	pDST RIF	MIRU-VNTR	TB treatment outcome	RAV frequency (%)
1	Male	47	Positive	142	Positive	Resistant	Susceptible	Single strain	Defaulted	<0.1 all loci
2	Male	28	Positive	Unknown	Positive	Resistant	Susceptible	Possibly mixed infection[b]	Completed	<0.1 all loci
3	Female	34	Positive	773	Positive	Resistant	Susceptible	Possibly mixed infection[b]	Completed	<0.1 all loci
4	Male	39	Positive	17	Negative	Resistant	Susceptible	Mixed infection[c]	Completed	17.01 *katG* 315ACC

[a]Abbreviations: pDST, phenotypic drug susceptibility testing; INH, isoniazid; RIF, rifampin; MIRU-VNTR, 24-locus mycobacterial interspersed repetitive units-variable number of tandem repeats; RAV, resistance-associated variants.
[b]Defined as the presence of ≥2 repeats at a single locus in the same sputum sample.
[c]Defined as the presence of ≥2 repeats at more than one locus in the same sputum sample.

by our SMOR assay may be responsible for clinical INH resistance, particularly among HIV-infected TB patients. While *katG* and *inhA* promoter mutations explain the majority of resistance to INH, a subset of INH-resistant clinical isolates do not carry these common mutations (15). Mutations in *katG* and *inhA* promoter genes have been shown to confer low fitness costs, allowing the mutant microbe to survive and propagate without negative selection pressure (16). It is possible that mutant *M. tuberculosis* strains with reduced fitness are more likely to survive and replicate in immunocompromised HIV-infected hosts, and these mutations may be missed by targeting only high-confidence, well-published RAVs. However, our results are limited by the small sample size of patients with drug-resistant TB. Additional research on a larger sample size of drug-resistant TB patients is needed to confirm our findings.

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