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Characterisation of Mycobacterium tuberculosis isolates lacking IS6110 in Viet Nam

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MYCOBACTERIOLOGY AND AEROBIC ACTINOMYCETES

Laboratory Evaluation of a Lateral-Flow Cell for Molecular Detection of First-Line and Second-Line Antituberculosis Drug Resistance

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ABSTRACT Despite the WHO's call for universal drug susceptibility testing for all patients being evaluated for tuberculosis (TB), a lack of rapid diagnostic tests which can fully describe TB resistance patterns is a major challenge in ensuring that all persons diagnosed with drug-resistant TB are started on an appropriate treatment regime. We evaluated the accuracy of the Akonni Biosystems XDR-TB TruArray and lateral-flow cell (XDR-LFC), a novel multiplex assay to simultaneously detect mutations across seven genes that confer resistance to both first- and second-line anti-TB drugs. The XDR-LFC includes 271 discrete three-dimensional gel elements with target-specific probes for identifying mutations in *katG*, *inhA* promoter, and *ahpC* promoter (isoniazid), *rpoB* (rifampin), *gyrA* (fluoroquinolones), *rrs* and *eis* promoter (kanamycin), and *rrs* (capreomycin and amikacin). We evaluated XDR-LFC performance with 87 phenotypically and genotypically characterized clinical *Mycobacterium tuberculosis* isolates. The overall assay levels of accuracy for mutation detection in specific genes were 98.6% for *eis* promoter and 100.0% for the genes *katG*, *inhA* promoter, *ahpC* promoter, *rpoB*, *gyrA*, and *rrs*. The sensitivity and specificity against phenotypic reference were 100% and 100% for isoniazid, 98.4% and 50% for rifampin (specificity increased to 100% once the strains with documented low-level resistance mutations in *rpoB* were excluded), 96.2% and 100% for fluoroquinolones, 92.6% and 100% for kanamycin, 93.9% and 97.4% for capreomycin, and 80% and 100% for amikacin. The XDR-LFC solution appears to be a promising new tool for accurate detection of resistance to both first- and second-line anti-TB drugs.

KEYWORDS extensively drug-resistant tuberculosis, drug susceptibility, isoniazid, rifampin, fluoroquinolone, kanamycin, capreomycin, amikacin, lateral-flow cell, tuberculosis

The WHO End TB Strategy calls for universal drug susceptibility testing (DST) for all patients being evaluated for tuberculosis (TB) (1). However, as recently as 2019 there were still major gaps in the global DST capacity: (i) only 51% of people with bacteriologically confirmed TB were tested for rifampin resistance (2), and (ii) among patients who were notified that they had multidrug-resistant TB (MDR-TB; defined as TB resistant to both first-line anti-TB drugs, isoniazid and rifampin), only 59% were tested for additional resistance to fluoroquinolones and second-line injectable agents (2) to

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TABLE 1 Resistance-conferring mutations covered by the XDR-LFC

Drug(s)	Resistance-conferring mutation(s)
Isoniazid	*katG* (S315N, S315R, S315T [ACC], S315T [ACA]), *inhA* promoter (−8a, −8c, −8g, −15t, −17t), *ahpC* promoter (−6t, −10a)
Rifampin	*rpoB* (Q513K, Q513L, M515I,[a] D516G, D516V, D516Y, S522L, H526C, H526D, H526L, H526N, H526R, H526Y, S531L, S531W, L533P)
Fluoroquinolones	*gyrA* (G88A, G88C, G88V, A90V, S91P, D94A, D94G, D94H, D94N, D94Y)
Kanamycin	*rrs* (A1401G, C1402T, G1484T), *eis* promoter (−10a, −12t, −14t, −15g, −37t)
Capreomycin	*rrs* (A1401G, C1402T, G1484T)
Amikacin	*rrs* (A1401G, C1402T, G1484T)

[a]*rpoB* M515I is reported as resistance conferring only in the presence of *rpoB* D516Y.

rule out extensively drug-resistant TB (XDR-TB; tuberculosis which is resistant to at least isoniazid and rifampin [e.g., MDR-TB] and 1 second-line fluoroquinolone and 1 second-line injectable, such as amikacin, kanamycin, or capreomycin). Additionally, in 2016 the WHO reported that 9 countries with more than 5,000 TB cases had no capacity to perform phenotypic DST (3), and only 6 out of the 40 countries (15%) with high TB or MDR-TB burdens had established a national continuous surveillance system for second-line drug resistance (3). While the second-line drugs kanamycin and capreomycin are no longer recommended for MDR-TB treatment by the WHO (4), resistance to these drugs is still part of the definition of XDR-TB, which can have up to 41% mortality, and thus is still critical for drug-resistant TB diagnostic decision trees and an important indicator of suitability for some of the novel regimens for drug-resistant TB still under trial (5).

Drug resistance (DR) in *Mycobacterium tuberculosis* is mainly due to single nucleotide polymorphisms (SNPs) in a small set of genes (6–8). The well-documented relationship(s) between phenotypic resistance and these resistance-conferring mutations has instigated a slow but transformative shift in DST methods from time-consuming cultured-based phenotypic methods to rapid sputum-based molecular DST (9). The Akonni Biosystems TruArray is a nucleic acid amplification test (NAAT) to rapidly diagnose and genotype *M. tuberculosis*, which integrates lateral-flow microfluidics with 271 discrete three-dimensional gel elements for target-specific probes in a small footprint (3.6 mm by 5.2 mm) (10–12). Asymmetric PCR amplification of targets occurs in the presence of the TruArray, simplifying the workflow and allowing for amplicon containment as a means of preventing workspace contamination (12–14). We previously reported on an MDR-TB TruArray MDR-TB and lateral-flow cell (LFC) which detects resistance to isoniazid and rifampin (11, 12), and here we report on the analytical performance of the Akonni Biosystems XDR-TB TruArray and lateral-flow cell (XDR-LFC), a novel multiplex XDR-TB assay to simultaneously detect mutations across seven genes that confer resistance to both first- and second-line anti-TB drugs.

MATERIALS AND METHODS

XDR-LFC description and molecular targets. The XDR-LFC workflow consists of two main procedures: (i) PCR amplification and hybridization to probes directly on the XDR-LFC using a flat block thermal cycler (ProFlex PCR system; Thermo Fisher Scientific, Waltham, MA) with a heat block adapted to fit eight XDR-LFCs and (ii) imaging of the processed microarrays. Microarray primers focusing on gene segments and probes detecting mutations in the *M. tuberculosis* genome known to confer phenotypic resistance to first- and second-line anti-TB drugs (Table 1; see also Table S1 in the supplemental material) were developed.

The XDR-LFC includes two probes to detect the presence of *M. tuberculosis* (IS*6110* and MPB64) and four probes to detect the presence of nontuberculous mycobacteria (NTM; using the gene *hsp65*) (Table S1). Additionally, the XDR-LFC contains four types of controls: (i) a Cy3 beacon for manufacturing quality control and positional reference, (ii) one control probe for an M13 internal positive amplification and inhibition control, (iii) one control probe for gene target *hsp65* to verify gene targets for NTM, and (iv) one control probe each for the seven *M. tuberculosis* drug-resistant genes to verify amplification of those genes.

The 11 PCR primer pairs were combined in a multiplexed master mix. Using an asymmetric PCR design, a Cy3-labeled primer from each primer pair was added at 10× to 17.5× the concentration of the unlabeled primer. The *katG*, *inhA* promoter, and *rpoB* PCR primer and microarray probe sequences were similar to those of the PCR primers and microarray probes for isoniazid and rifampin used in a previous study (11), while primers for *ahpC* promoter, *gyrA*, *rrs*, and *eis* promoter were new (Table S1). The resulting Cy3-labeled amplicons ranged from 92 to 139 nucleotides in length for the seven gene regions

examined. All *M. tuberculosis* drug-resistant gene probes were paired (e.g., one probe signaled the wild type [WT] and the second probe signaled the presence of the SNP of interest [MUT]). Each XDR-LFC included drug-resistant locus probes in triplicate.

Selection of *M. tuberculosis* strains. Eighty-two strains with M/XDR-TB phenotypes and five pan-susceptible clinical *M. tuberculosis* strains were selected from our archive housed at University of California, San Diego, of 1,946 strains collected from four countries (India, Republic of Moldova, Philippines, and South Africa) for which previously characterized genetic sequences and phenotypic DST results were available (15). Strain selection for this study was focused on maximizing *M. tuberculosis* genomic diversity within the context of the resistance mutations covered by the XDR-LFC (Table 1) while ensuring agreement between sequences if multiple sequencing platforms were used.

Selection of NTM strains. The XDR-LFC was designed to be specific for *M. tuberculosis*. We evaluated potential cross-reactivity with NTM by including an analysis of seven NTM species: *M. abscessus* (ATCC 19977D-5), *M. avium* (ATCC 25291), *M. gordonae* (ATCC 14470), *M. kansasii* (ATCC 12478), *M. malmoense* (ATCC 29571), *M. marinum* (ATCC BAA 53D-5), and *M. smegmatis* (ATCC 700084D-5).

DNA extraction and quantification. There was enough DNA previously extracted to run experiments for 76 isolates. Briefly, those isolates were grown on Löwenstein-Jensen medium, killed by exposure to ethanol and heat, and lysed. DNA was extracted using NaCl, cetyltrimethylammonium bromide (CTAB), and chloroform-isoamyl alcohol (16, 17). Eleven isolates were cultured and extracted by crude extraction (heat kill and sonication) specifically for this research. The XDR-LFC detection system is optimized to detect 1 pg of *M. tuberculosis* DNA in a clinical sample. We normalized the extracted DNA concentration for all *M. tuberculosis* isolates based on the NanoDrop 2000 spectrophotometer and then diluted the normalized DNA to 45 fg/μl in order to obtain approximately 1 pg of *M. tuberculosis* DNA in a 22-μl volume, the input volume for the XDR-LFC.

XDR-LFC methods and automated imaging and data analysis. Twenty-two microliters of normalized DNA was added to 58 μl of PCR mix, and reaction mixtures were added to the XDR-LFC chambers, which were placed on a ProFlex block thermal cycler and run using the following parameters: initial denaturation at 89°C for 5 min, 30 cycles of denaturation at 89°C for 50 s, touchdown annealing at 61 to 55°C for 65 s, and extension at 65°C for 35 s, followed by 20 cycles of denaturation at 89°C for 50 s, annealing at 55°C for 65 s, and extension at 65°C for 35 s and a final extension at 65°C for 3 min. After thermocycling, XDR-LFCs were washed and centrifuged to remove any residual liquid before imaging as per manufacturer instructions.

Washed microarrays were imaged postamplification for 100 ms on a prototype Akonni Dx2000 imager (12). The average and median integrated signal intensity (ISI) for each set of triplicate probes were calculated and the average ISI was used unless the absolute value of the difference between the average and median ISIs was >0.3; then, the median ISI was used. ISI was then normalized against the background image noise.

Positive detection of the IS*6110* target was set at a normalized ISI value of ≥ 20, and values above this triggered the automated analysis of gene detection probes. Prior experimentation based on testing samples with known mutations and a variety of DNA concentrations determined that there was some variation in gene amplification efficiency and if gene detection probes for *katG*, *inhA* promoter, and *rpoB* had a normalized ISI value of <10, the test was deemed indeterminate, while gene detection probes for *ahpC* promoter, *gyrA*, *rrs*, and *eis* promoter had a normalized ISI value of <5 for indeterminate calls. The wild-type and mutant probe discrimination ratio (D) was calculated where $D = (ISI_{WT} - ISI_{MUT})/(ISI_{WT} + ISI_{MUT})$, where ISI_{WT} is the normalized ISI value for the wild-type probe and ISI_{MUT} is the normalized ISI value for the mutant probe. Values of D less than -0.1 were interpreted as a mutation, while D values of >0.1 were reported to be the wild type. For accurate detection of *gyrA* D94A SNP, optimized thresholds of 0.2 and -0.2 were selected. Values of D between thresholds were deemed to be indeterminate results. While not a sequencing device, the algorithm and software report the SNP with the lowest negative D as the most likely mutation found except in *rpoB*, in which up to three mutations are reported to account for double/triple mutations.

Reference phenotyping methods. MGIT liquid phenotypic DST results for all *M. tuberculosis* isolates used in this study have been described in detail previously (15) and were based on WHO-recommended critical drug concentrations in place during isolate collection (18, 19): isoniazid, 0.1; rifampin, 1.0; moxifloxacin, 0.25, and ofloxacin, 2.0 (combined to represent fluoroquinolones); kanamycin, 2.5; capreomycin, 2.5; and amikacin, 1.0 (all concentrations in micrograms per milliliter) (15).

Reference genotyping methods. Sequencing results for *M. tuberculosis* strains were obtained previously with pyrosequencing, using a PyroMark Q96 (PSQ; Qiagen, Valencia, CA) (15, 20), Sanger sequencing (SAN) (8), and/or whole-genome sequencing on a Pacific Biosciences (PacBio) single-molecule, real-time sequencer (Pacific Biosciences, Menlo Park, CA) (16, 17). Any isolate that was sequenced using more than one sequencing method and had conflicting sequence results for a mutation of interest were deemed nonconsensus (NC), and the entire NC gene for that isolate was censored for subsequent analyses.

Quantitative PCR. After the XDR-LFC runs were completed, we analyzed the DNA quantity in each diluted DNA sample using quantitative PCR (qPCR). qPCR was performed in duplicate using the single-copy *rpoB* gene as a target on a LightCycler 480 instrument (Roche LifeScience). The PCR parameters were as follows: initial denaturation at 95°C for 10 min and 45 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 30 s, and extension at 72°C for 45 s. A single 25-μl reaction mixture contained 5 μl of DNA, 1× LightCycler FastStart DNA Master HybProbe buffer and enzyme (Roche), 2.5 mM $MgCl_2$, 1.35 μM forward primer, 0.45 μM reverse primer, and 25 nM fluorescent probe. An *rpoB*-specific primer and probe set was developed (forward primer, 5′-GGCGATCACACCGCAGACGTT;

TABLE 2 Summary of XDR-LFC results against a genomic reference of clinical isolates

Gene	XDR-LFC	No. of isolates with reference genome		% Accuracy	% sensitivity (95% CI)	% specificity (95% CI)
		Mutation	WT			
katG	Mutation	52	0	100	100	100
	None	0	8		(93.2–100)	(63.1–100)
inhA promoter	Mutation	38	0	100	100	100
	None	0	35		(90.7–100)	(90–100)
ahpC promoter	Mutation	2	0	100.0	100	100.0
	None	0	64		(15.8–100)	(94.4–99.6)
rpoB	Mutation	65	0	100	100	100
	None	0	6		(94.5–100)	(54.1–100)
gyrA	Mutation	49	0	100	100	100
	None	0	21		(92.7–100)	(83.9–100)
rrs	Mutation	30	0	100	100	100
	None	0	41		(88.4–100)	(91.4–100)
eis promoter	Mutation	20	0	98.6	95.2	100.0
	None	1	50		(76.2–99.9)	(92.9–100)

reverse primer, 5′-CACGCTCACGTGACAGACCGCCG; and probe, 5′-6-carboxyfluorescein [FAM]- ACCACC GGCCGGATGTTGATC-black hole quencher 1 [BHQ1]) targeting a section of the *rpoB* gene. For relative quantification, a dilution series of H37Ra genomic DNA (from 10 pg/μl to 10 fg/μl) was run to generate the standard curve. *M. tuberculosis* DNA was considered detected in the sample if the threshold cycle (C_T) value for both PCR replicates was $\leq$40.

Statistical analyses. Statistical analyses were conducted in R (21) using the library epiR (v1.0-14).

RESULTS

A total of 94 XDR-LFCs were evaluated: 87 with DNA extracted from cultured *M. tuberculosis* clinical isolates and 7 with DNA extracted from cultured NTM species.

Assay specificity for *M. tuberculosis*. *M. tuberculosis* was detected in the extracted DNA normalized to ~1 pg from 73 (83.9%) of the 87 clinical isolates. The XDR-LFC did not indicate detection of *M. tuberculosis* in the seven NTM samples, demonstrating that the *M. tuberculosis* detection probe in the XDR-LFC had low cross-reactivity with NTM. However, the XDR-LFC detected NTM in only four NTM samples (*M. abscessus*, *M. malmoense*, *M. marinum*, and *M. smegmatis*) and not in the other three samples (*M. avium*, *M. gordonae*, and *M. kansasii*), indicating potential nonoptimal sensitivity for NTM species.

Assay performance against genotypic standard. Eleven of the 24 (46%) codons/nucleotides of interest included on the XDR-LFC were tested against more than 5 isolates, while 6 codons/nucleotides of interest were tested against more than 20 isolates (Table S2). Nine isolates had NC sequencing results between PSQ, SAN, and PacBio for *katG*, one isolate had NC results for *rpoB*, two isolates for *gyrA*, two isolates for *rrs*, and one isolate for *eis* promoter (Table S3). Where specific genes in specific *M. tuberculosis* isolates had NC genetic results among the three reference sequencing methods, we removed that whole gene from the genotypic analysis. We had no *M. tuberculosis* isolates with SNPs at *rrs* 1402 or *eis* promoter −15 in our archive of clinical samples; thus, these two XDR-LFC mutation probes could not be tested against clinical resistant strains.

Overall, the assay accuracy for mutation detection in specific genes ranged from 98.6% for *eis* promoter to 100.0% for the genes *ahpC* promoter, *katG*, *inhA* promoter, *rpoB*, *gyrA*, and *rrs* (Table 2). One miscalled mutation was observed for which the XDR-LFC did not detect an apparent −37t *eis* promoter mutation that was reported in the SAN reference sequencing data (isolate 3-0121, MGIT phenotypically susceptible to

TABLE 3 Phenotypic resistance patterns of the 86 *Mycobacterium tuberculosis* isolates tested

Drug(s)[a]	No. of isolates with indicated resistance phenotype	
	Resistant	Susceptible
Isoniazid	81	5
Rifampin	72	14
Fluoroquinolones	61	25
Kanamycin	57	29
Capreomycin	36	50
Amikacin	43	43

[a]Liquid MGIT DST critical concentrations for each drug were as follows (all concentrations in micrograms per milliliter): isoniazid, 0.1; rifampin, 1.0; moxifloxacin, 0.25, and ofloxacin, 2.0 (combined to represent fluoroquinolones); kanamycin, 2.5; capreomycin, 2.5; and amikacin, 1.0.

kanamycin). No PSQ or PacBio data were available for this sample for verification of the mutation in the reference data in order to additionally confirm the presence of this mutation. In this isolate, the XDR-LFC −37t *eis* promoter probe discrimination ratio was strongly positive (0.94), indicating that this was a valid LFC wild-type call, and the ISI values for the three replicate probes were well above background noise, suggesting that the SNP may have been misclassified in our reference sequencing data. No other isolate had a mutation in *eis* promoter at the −37 position.

The output of the XDR-LFC algorithm, which reports the most likely (i.e., suspected) mutation(s), was concordant with sequencing for 304 of 308 specific SNP mutations (Table S4), and none of the four discordant SNP mutations resulted in incorrect phenotypic calls. In isolates 4-0013 and 4-0023, the XDR-LFC reported a −6t mutation in the *ahpC* promoter, while PacBio sequencing results indicated a −6a mutation in the *ahpC* promoter (note that while the −6t *ahpC* promoter is included in the XDR-LFC, the −6a mutation is not). Two other errors occurred within *katG* which also did not affect resistance calls. In isolate 3-0119, the XDR-LFC detected an S315T mutation, while SAN sequencing indicated an S315N *katG* mutation, and in isolate 4-0013, the XDR-LFC detected an S315R mutation, while SAN sequencing indicated an S315T *katG* mutation. The most likely mutation is reported as the probe discrimination ratio with the lowest negative value, and while the reference mutation discrimination ratio was negative, it was not the minimum; values of *D* were as follows: S315N = −0.59, **S315T (ACA)** = −0.81, and S315T (ACC) = −0.20 for isolate 3-0119 and S315N = indeterminate, S315T (ACC) = −1.15, S315T (ACA) = −1.00, and **S315R** = −1.25 for isolate 4-0013 (mutations in bold are XDR-LFC-reported SNPs).

Assay performance against phenotypic standard. The phenotypic DST results for the 86 isolates are shown in Table 3. One isolate (4-0023) had no growth during microbial revival, and so phenotypic DSTs were not performed. Five isolates were pan-susceptible (5.7%), 4 were isoniazid resistant (4.6%), 10 were MDR-TB (11.5%), 8 were MDR with additional fluoroquinolone resistance (9.2%), 6 were MDR with additional injectable resistance (6.9%), 48 were XDR-TB (55.2%), and 5 had other resistant phenotypes (5.8%).

The overall XDR-LFC performance on DNA extracted from clinical *M. tuberculosis* isolates is shown in Table 4. XDR-LFC accuracy ranged from 100% for isoniazid to 88.9% for amikacin. The sensitivity and specificity of the XDR-LFC were 100% and 100% for isoniazid, 98.4% and 50% for rifampin, 96.2% and 100% for fluoroquinolones, 92.6% and 100% for kanamycin, 93.9% and 97.4% for capreomycin, and 80% and 100% for amikacin, respectively.

DNA extractions were diluted to approximately 1 pg/22 μl during normalization. Postrun analysis by qPCR indicated that only 24 samples (27.6%) had DNA concentrations of $\geq$1 pg per 22-μl reaction mixture, which met the XDR-LFC design specifications for *M. tuberculosis* detection. Of the 63 XDR-LFC runs for which the input DNA concentration was $<$1 pg of DNA per reaction mixture, the XDR-LFC was still able to detect *M. tuberculosis* in 49 (77.8%) of the XDR-LFCs, with a mean DNA concentration

TABLE 4 Summary of XDR-LFC results against MGIT phenotypic reference of clinical isolates[a]

Drug(s)	XDR-LFC	No. of isolates with phenotypic DST result		Total % accuracy	% sensitivity (95% CI)	% specificity (95% CI)
		R	S			
Isoniazid	Mutations detected	64	0	100	100	100
	None detected	0	5		(94.4–100.0)	(47.8–100.0)
Rifampin	Mutations detected	61	5	91.7	98.4	50.0
	None detected	1	5		(91.3–100.0)	(18.7–81.3)
Fluoroquinolones	Mutations detected	50	0	97.2	96.2	100
	None detected	2	19		(86.8–99.5)	(82.4–100)
Kanamycin	Mutations detected	50	0	94.4	92.6	100
	None detected	4	18		(82.1–97.9)	(81.5–100)
Capreomycin	Mutations detected	31	1	95.8	93.9	97.4
	None detected	2	38		(79.8–99.3)	(86.5–99.9)
Amikacin	Mutations detected	32	0	88.9	80	100
	None detected	8	32		(64.4–90.9)	(89.1–100)

[a]Liquid MGIT DST critical concentrations for each drug were as follows (all concentrations in micrograms per milliliter): isoniazid, 0.1; rifampin, 1.0; moxifloxacin, 0.25, and ofloxacin, 2.0 (combined to represent fluoroquinolones); kanamycin, 2.5; capreomycin, 2.5; and amikacin, 1.0. R, resistant; S, susceptible.

of 0.44 pg of DNA per reaction mixture, while the 14 XDR-LFCs that showed *M. tuberculosis* to be not detected had a mean DNA concentration of only 0.07 pg of DNA per reaction mixture. In aggregate, *M. tuberculosis* was detected in 73 XDR-LFCs, with a mean DNA concentration of 2.39 per 22-μl reaction mixture.

DISCUSSION

In this lab accuracy study, we evaluated a novel Akonni Biosystems XDR-TB TruArray which was challenged with a diverse set of clinical isolates from a globally representative *M. tuberculosis* strain repository. The Akonni Biosystems XDR-TB TruArray demonstrated excellent performance with regard to its ability to accurately detect a large diversity of resistance-conferring mutations found in clinical XDR-TB phenotypes. The XDR-LFC was designed to detect *M. tuberculosis* drug resistance genes when the input DNA is at least 1 pg per reaction, which is roughly equivalent to being able to successfully get a result from clinical samples with acid-fast bacillus (AFB) smear status that is "scanty" or above. When challenged with contrived samples with $<$1 pg per reaction, results from the XDR-LFC in this study indicated that it is likely to still perform adequately down to $\sim$0.5 pg of DNA per reaction, but future studies should include a systematic estimate of the limit of detection for this assay, as it is likely to fall somewhere between 0.5 pg and 1 pg of input DNA per reaction.

The Akonni Biosystems XDR-TB TruArray had no cross-reactivity with the *M. tuberculosis*-specific probes when challenged with seven NTM species. As the focus of the array was to characterize drug resistance in *M. tuberculosis*, the lack of cross-reactivity with NTM reduces the likelihood of future false-positive results driven by NTM presence. However, the probes utilized for detecting the presence of NTM need to be further optimized for detection of NTMs, as only four of the seven species examined were detected with this assay.

The sole performance issue with the array was the less-than-optimal rifampin specificity (50%, with a 95% confidence interval [CI] of 18.7% to 81.3%). However, it appears that this low apparent specificity was driven by five *M. tuberculosis* isolates that appeared to give false-positive resistance results (Table 4). Sequencing data revealed that all five isolates contained mutations in the *rpoB* gene, which are well documented to confer low-level resistance, with MICs that fall below the WHO-endorsed critical concentration for rifampin MGIT DST. Strains with these *rpoB* mutations therefore can appear to have rifampin-susceptible phenotypes by MGIT DST but usually show as

resistant on 7H10 agar DST or with an extended MGIT DST protocol that accounts for continued growth due to low-level resistance (22). Specifically, the XDR-LFC correctly identified three *M. tuberculosis* isolates that harbored mutations in *rpoB* D516Y, one that had a H526C mutation, and one that had an H526L mutation. If these five isolates with low-level resistance mutations are excluded, rifampin specificity increased to 100%. The one rifampin false-negative result was phenotypically resistant but on sequencing showed as wild type for *rpoB* on all three sequencing platforms, suggesting that the XDR-LFC correctly identified it as such despite its apparent susceptible phenotype—indicating a likely error in the reference phenotype of this strain. In the case of the two fluoroquinolone false-negative results, both isolates harbored *gyrA* E21Q and G668D mutations (as determined by PacBio reference sequencing), which have been previously shown to be associated with phenotypic resistance to ofloxacin (23, 24) but are currently not considered markers of fluoroquinolone resistance (25) and were therefore not included in the XDR-LFC probe design.

In the case of the four false-negative results obtained for kanamycin, three isolates were wild type in *rrs* and *eis* promoter sequences and one was wild type in *rrs* but we detected an NC mutation in *eis* promoter (SAN, −14t; PacBio, WT; PSQ, not done). In the case of the two false negatives for capreomycin, both isolates were wild type in *rrs*. The isolate with the one false-positive result for capreomycin had a mutation at 1401G in *rrs*. In the case of the eight false-negative results for amikacin, all isolates were wild type in *rrs*, while six harbored *eis* promoter mutations at −14t, one had an *eis* promoter mutation at −10A, and one was NC in *eis* promoter (SAN, −14T; PacBio, WT; PSQ, not done).

Conclusions. The XDR-LFC assay has several strengths. (i) It includes both IS*6110* and MPB64 as targets to identify *M. tuberculosis*, which allows for detection of *M. tuberculosis* in clinical samples when IS*6110* is not present, which can vary from <1% in the United States (26) to 4% in Viet Nam (27) and 11% in India (28). (ii) It provides a comprehensive picture of drug resistance, as it is multiplexed to simultaneously test for mutations across seven genes which confer *M. tuberculosis* resistance to first- and second-line anti-TB drugs, including several clinically relevant variants of the same codon (e.g., *katG* S315N, S315R, S315T [ACC], and S315T [ACA]). (iii) Test implementation and data analysis are simple. After DNA is extracted, the sample is introduced into a single microfluidic test slide that requires only two fluidic steps for amplification, hybridization, washing, and imaging through an intact device (i.e., without disassembly), allowing for amplicon containment to prevent PCR contamination. Additionally, data analysis of the array is completely automated, including spot recognition, intensity value extraction, background correction, genotype ratio calculations, *M. tuberculosis* detection, and drug resistance calls. (iv) The analytical performance of the XDR-LFC is appropriately aligned with the WHO high-priority target product profiles (TPP) for new TB diagnostics by exceeding minimum TPP sensitivity against phenotypic DST (>95% for rifampin and >90% for fluoroquinolones), and if strains with low-level resistance mutations in *rpoB* are excluded, it exceeded the minimum TPP specificity of ≥98% against phenotypic DST. For diagnostic specificity (except for *ahpC* promoter), the XDR-LFC exceeds the minimum TPP of ≥99.7% against genetic sequencing (29).

In conclusion, the XDR-LFC assay is an effective method for identifying *M. tuberculosis* and simultaneously detecting multiple drug resistance-conferring mutations from TB isolates. The entire procedure, from extraction of DNA to reporting results, can be accomplished within 8 h using a protocol that can be integrated into the existing workflow of diagnostic or public health laboratories. Finally, the XDR-LFC assay is a flexible technology which runs six clinical samples simultaneously, increasing test throughput, and represents a significant technological advancement in the detection of complex drug-resistant *M. tuberculosis* strains from direct clinical samples.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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