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Clinical Evaluation of the XDR-LFC Assay for the Molecular Detection of Isoniazid, Rifampin, Fluoroquinolone, Kanamycin, Capreomycin, and Amikacin Drug Resistance in a Prospective Cohort

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ABSTRACT While the goal of universal drug susceptibility testing has been a key component of the WHO End TB Strategy, in practice, this remains inaccessible to many. Rapid molecular tests for tuberculosis (TB) and antituberculosis drug resistance could significantly improve access to testing. In this study, we evaluated the accuracy of the Akonni Biosystems XDR-TB (extensively drug-resistant TB) TruArray and lateral-flow-cell (XDR-LFC) assay (Akonni Biosystems, Inc., Frederick, MD, USA), a novel assay that detects mutations in seven genes associated with resistance to antituberculosis drugs: *katG*, the *inhA* promoter, and the *ahpC* promoter for isoniazid; *rpoB* for rifampin; *gyrA* for fluoroquinolones; *rrs* and the *eis* promoter for kanamycin; and *rrs* for capreomycin and amikacin. We evaluated assay performance using direct sputum samples from 566 participants recruited in a prospective cohort in Moldova over 2 years. The sensitivity and specificity against the phenotypic reference were both 100% for isoniazid, 99.2% and 97.9% for rifampin, 84.8% and 99.1% for fluoroquinolones, 87.0% and 84.1% for kanamycin, 54.3% and 100% for capreomycin, and 79.2% and 100% for amikacin, respectively. Whole-genome sequencing data for a subsample of 272 isolates showed 95 to 99% concordance with the XDR-LFC-reported suspected mutations. The XDR-LFC assay demonstrated a high level of accuracy for multiple drugs and met the WHO's minimum target product profile criteria for isoniazid and rifampin, while the sensitivity for fluoroquinolones and amikacin fell below target thresholds, likely due to the absence of a *gyrB* target in the assay. With optimization, the XDR-LFC shows promise as a novel near-patient technology to rapidly diagnose drug-resistant tuberculosis.

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

Tuberculosis (TB) remains a major cause of morbidity and mortality worldwide, ranking second behind coronavirus disease 2019 (COVID-19) among causes of death from a single infectious agent (1). The goal of the WHO End TB Strategy is to reduce the incidence of TB by 90% and TB deaths by 95% by 2035, and one of the key

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components central to this strategy is universal drug susceptibility testing (DST) (2). Unfortunately, access to diagnostics and DST for bacteriological confirmation of TB cases remains limited, with only 63% of pulmonary TB cases being bacteriologically confirmed in 2021 (1). Culture-based methods remain the traditional gold standard for phenotypic DST (pDST) but are constrained by the need to wait weeks to months to obtain results. To reduce this delay, there has been a call for the expansion of the use of rapid molecular testing for the detection of TB and drug resistance, yet in 2021, only 38% of newly diagnosed TB cases received WHO-recommended rapid molecular testing for TB detection (1). There are now multiple WHO-approved rapid platforms for DST, with drug resistance information, costs, and complexity varying by platform (3). Until recently, the most accessible and widespread rapid molecular testing systems have tested for rifampin resistance alone; however, platforms for multidrug-resistant TB (MDR-TB) and second-line drug testing are seeing increased investment and usage given the pressing need for practical next-generation rapid DST for MDR-TB.

Updated 2021 WHO TB drug resistance classifications now include monoresistant TB, rifampin-resistant TB (RR-TB), polyresistant TB, MDR-TB, pre-extensively drug-resistant TB (pre-XDR-TB), and extensively drug-resistant TB (XDR-TB) (4). While the WHO no longer defines TB drug resistance using aminoglycoside second-line injectable drugs (SLIDs), nor does it recommend their use for the treatment of MDR-TB, with the exception of amikacin, it has taken time to transition to all-oral regimens, and SLIDs remain in use for treatment (5, 6). It is therefore important that comprehensive DST be available until they are fully transitioned out of use and for surveillance purposes.

Genotypic molecular assays for DST have become increasingly promising given the well-documented relationships between identified resistance-conferring mutations and phenotypic resistance and the recent publication of the first WHO *Mycobacterium tuberculosis* mutation catalog in 2021 (7). The novel Akonni TruArray MDR-TB and lateral-flow-cell platform (Akonni Biosystems, Inc., Frederick, MD, USA) has been previously described (8–11) and can be used to rapidly diagnose and genotype *M. tuberculosis* from sputum samples. We have previously evaluated the analytical laboratory performance of the expanded Akonni Biosystems XDR-TB TruArray and lateral-flow cell (XDR-LFC) for the detection of mutations across seven genes associated with resistance to isoniazid, rifampin, fluoroquinolones, kanamycin, capreomycin, and amikacin using clinical isolates, demonstrating its proof of principle for use for the rapid detection of MDR-TB, pre-XDR-TB, and SLID-resistant TB on a single platform (12). In that study, the XDR-LFC assay was evaluated using archived clinical *M. tuberculosis* isolates that had been phenotypically and genotypically characterized (12). This study expands on the previous development work by evaluating the performance of the XDR-LFC assay using clinical sputum samples collected from participants prospectively as part of a large clinical cohort study.

MATERIALS AND METHODS

Participant enrollment. Participants were enrolled as part of a prospective cohort study of patients considered at risk for drug-resistant TB, using a protocol described in detail previously by Hillery et al. (13). Briefly, enrollment was conducted at multiple regional TB clinics in the Republic of Moldova. Participants were eligible for enrollment if they had presumptive or proven clinically active TB disease and were at risk of having drug-resistant TB. The clinical diagnosis of active TB disease was classified as bacteriologically confirmed by sputum acid-fast bacillus (AFB) smear positivity or Xpert MTB/RIF assay positivity within 7 days of enrollment or as clinically presumptive TB disease. Enrolled participants were considered at risk for drug-resistant TB if they had previously been treated for at least 1 month with antitubercular medications for a previous TB episode, had failed standard TB treatment, had close contact with a known drug-resistant TB case, had been diagnosed with rifampin-resistant TB within the past 30 days, or had previously been diagnosed with MDR-TB and were suspected of failing a standard MDR-TB treatment regimen. Individuals were excluded if they were unable to provide at least 8.5 mL of sputum at the time of enrollment, had started treatment for their current TB episode more than 14 days before enrollment, were less than 5 years of age, were pregnant, were institutionalized, or were imprisoned. Individuals who could not produce 8.5 mL of sputum initially were requested to attempt a second sample after waiting for 2 h, and the pooled sputum volume was used to determine inclusion. Sputum samples, questionnaires, and clinical data were collected from the participants. All participants completed written informed consent, and the study was approved by the University of California, San Diego,

TABLE 1 Resistance-conferring mutations included in the XDR-LFC assay

Drug(s)	Gene	Resistance-conferring mutations
Isoniazid	*katG*	S315N, S315R, S315T (ACC), S315T (ACA)
	inhA promoter	−8a, −8c, −8g, −15t, −17t
	ahpC promoter	−6t, −10a
Rifampin	*rpoB*	Q432K, Q432L, M434I,[a] D435G, D435V, D435Y, S441L, H445C, H445D, H445L, H445N, H445R, H445Y, S450L, S450W, L452P
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* M434I is reported as a resistance-conferring mutation only in the presence of *rpoB* D435Y.

Human Research Protections Program (project number 161864) and the Ethics Committee of the Phthisiopneumology Institute Chiril Draganiuc.

Akonni Biosystems XDR-LFC description. The Akonni Biosystems XDR-LFC assay is in development and is not yet commercially available, being currently limited to research use only, and methods, specifications, and targets have been described previously in detail by Catanzaro et al. (12). In brief, PCR amplification and hybridization to probes directly on the XDR-LFC are done within the lateral-flow-cell system using a flat-block thermal cycler (ProFlex PCR system; Thermo Fisher Scientific, Waltham, MA, USA), followed by imaging of the processed microarrays. Probes for IS*6110* and MPB64 are used to detect the presence of *M. tuberculosis*, and four probes for the *hsp65* gene are used to detect nontuberculous mycobacteria (NTM). Probes present on the XDR-LFC are included to detect mutations in *M. tuberculosis* associated with phenotypic resistance to isoniazid, rifampin, fluoroquinolones, kanamycin, capreomycin, and amikacin, with targets listed in Table 1. After processing the samples with the XDR-LFC assay, the DNA quantity in each diluted DNA sample was analyzed by quantitative PCR (qPCR) using the single-copy *rpoB* gene as a target on a LightCycler 480 instrument (F. Hoffmann-La Roche, Basel, Switzerland) (12). The DNA quantities for all samples were determined by taking the means from duplicate qPCR runs.

XDR-LFC sample preparation and workflow. Following collection, 1 mL of raw sputum was aliquoted and stored at −70°C until it underwent liquefaction and heat kill processing followed by DNA extraction and purification using the Akonni TruTip automated workstation (14). This portable benchtop system performed automated sample liquefaction and homogenization using magnetically induced vortexing, lysis using heating strips at 90°C, and DNA extraction and purification using the Akonni TruTip nucleic acid purification kit. The DNA extraction and purification steps were performed in Moldova. Samples were then stored at −20°C and shipped to the University of California, San Diego, for further processing due to reagent availability constraints in Moldova. The workflow for XDR-LFC sample processing was conducted as previously described (12, 15). Study staff performing index XDR-LFC testing in San Diego, CA; reference pDST testing in Moldova; and whole-genome sequencing (WGS) testing in Flagstaff, AZ, were each blind to the results of the other staff.

Reference phenotyping methods. A separate 2.5-mL aliquot of the raw sputum sample from each participant was processed for decontamination and sediment concentration, and 0.5 mL of the sediment was used for pDST in liquid culture using the Bactec MGIT 960 platform (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Preliminary identification of the organism was done by Ziehl-Neelsen staining and blood agar plate methods, and confirmatory identification was done by MPB64 antigen detection. Isolates underwent drug susceptibility testing for multiple antitubercular agents using the WHO-recommended critical drug concentrations of 0.1 μg/mL isoniazid, 1.0 μg/mL rifampin, 0.25 μg/mL moxifloxacin, 1.5 μg/mL levofloxacin, 2.5 μg/mL kanamycin, 2.5 μg/mL capreomycin, and 1.0 μg/mL amikacin (16, 17). Critical drug concentrations were based on updated 2012 WHO interim recommendations, except for moxifloxacin, for which the lower critical threshold of 0.25 μg/mL from 2008 and 2018 guidelines was used as an indication of resistance.

Genomic characterization methods. A convenience subset of culture-positive samples was processed for WGS. From cultures, DNA was extracted using NaCl, cetyltrimethylammonium bromide, and chloroform-isoamyl alcohol. The extracted DNA samples were stored at −20°C and processed for WGS at the Translational Genomics Research Institute (Flagstaff, AZ, USA), where library preparation was done using NEBNext Ultra II DNA library prep kits (New England BioLabs, Ipswich, MA), and sequencing was performed on the Illumina NextSeq platform (Illumina, Inc., San Diego, CA, USA). The WGS output was then processed using the TBProfiler (version 2.8.12) pipeline to identify resistance-conferring mutations in the *katG*, *inhA* promoter, *ahpC* promoter, *rpoB*, *gyrA*, *rrs*, and *eis* promoter genes (18). The sequencing

data were assessed for quality, and samples with a mapped percentage of 95% or lower for a gene were censored from WGS analyses involving that gene. Samples with missing sequencing data at positions corresponding to mutations of interest with the XDR-LFC were censored from analyses involving those mutations.

Statistical analyses. The performance of the XDR-LFC was evaluated using pDST as the reference standard. XDR-LFC results for genes that were indeterminate for an isolate were censored from analyses involving that specific gene. Specifically, if the XDR-LFC detected any resistance-conferring mutations for a drug, the isolate was reported as being resistant to that drug, regardless of the presence of any indeterminate result for another gene(s) associated with resistance-conferring mutations in that drug. Conversely, if the XDR-LFC had any indeterminate results within any of the resistance-conferring mutations for a drug, even if the rest of the resistance-conferring mutations for the other gene(s) were deemed to be of the wild type, the isolate was considered indeterminate for that drug. In concordance with previous studies (19, 20), samples for which the XDR-LFC and pDST results were discordant for two or more drug classes (isoniazid, rifampin, fluoroquinolones, or aminoglycosides) were deemed to likely reflect sample processing or labeling errors and were censored from the final analysis. Statistical analyses were conducted using Stata 17 (StataCorp LP, College Station, TX, USA) (21). Confidence intervals (CIs) were calculated using the Wilson score method.

Data availability. Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/sra/PRJNA930488 (BioProject accession: PRJNA930488).

RESULTS

Study participants. A total of 647 participants were screened at three sites in the Republic of Moldova from 19 July 2017 to 2 July 2019. These clinical sites included two hospitals specializing in the care of patients diagnosed with TB (n = 272 and n = 242) and one municipal hospital (n = 125). Of those screened, 3 individuals were deemed ineligible, 10 did not provide consent and were excluded, and 5 initially enrolled but subsequently voluntarily withdrew from the study. Nine samples demonstrated discordant XDR-LFC and pDST results for two or more drug classes, and these participants were censored from the analyses (details are available in Table S1 in the supplemental material). The remaining 620 participants were included in the study. Questionnaire responses, clinical data, and sputum samples were collected from all participants and processed according to the study protocols (Table 2 and Fig. 1) (13).

Assay performance for detection of *Mycobacterium tuberculosis*. Of the 620 participants recruited to the study and included in the final analysis, the XDR-LFC was run on samples from 557 participants, with 63 being excluded due to delays in sample transport from Moldova. Of the 557 samples run on the XDR-LFC, all had valid results by internal controls. The XDR-LFC detected *M. tuberculosis* in 331 (59.4%) of the 557 samples included, with no *M. tuberculosis* DNA being detected in the other 226 (40.6%) samples. The XDR-LFC detected concurrent *M. tuberculosis* and NTM in one sample and NTM alone in two samples.

The XDR-LFC assay was optimized for the detection of *M. tuberculosis* at a concentration of 1 pg of input DNA per reaction mixture. Of the tested samples, the assay detected *M. tuberculosis* with DNA concentrations above this threshold in 219 samples. Additionally, the assay was able to detect *M. tuberculosis* in 112 samples despite detecting below 1 pg of DNA, and *M. tuberculosis* DNA amounts and detection increased as the sputum AFB smear grade increased (Table 3). In samples in which the XDR-LFC did not detect *M. tuberculosis*, the average and median DNA concentrations were below 1 pg across all smear grades.

Of the 557 samples run on the XDR-LFC, the XDR-LFC detected *M. tuberculosis* in 310 samples (74.3%) of the 417 samples that were culture positive by MGIT. Of these 417 culture-positive samples, the XDR-LFC detected *M. tuberculosis* in 55 (37.2%) of 148 samples with negative sputum AFB smears, 39 (86.7%) of 45 samples with scanty AFB smears, and 216 (96.4%) of 224 samples with sputum AFB smear grades of 1+ or higher. Of the 159 MGIT culture-negative samples, the XDR-LFC detected *M. tuberculosis* in 21 (15.0%) of the 140 samples on which the XDR-LFC was run.

Assay performance for detection of resistance-conferring mutations against a phenotypic standard. XDR-LFC results were available for 310 of the culture-positive samples, and the pDST results for these 310 isolates are shown in Table 4. Of these, 38 (12.3%) isolates were pansusceptible, 7 (2.3%) were isoniazid monoresistant, none

TABLE 2 Participant characteristics[a]

Characteristic	Value (n = 620)[b]
No. of male participants/total no. of participants (%)	491/618 (79.5)
Age at recruitment	
Median age (yrs) (IQR)	42 (34–51)
No. of patients in age group (yrs) (%)	
5–19	7 (1.1)
20–39	256 (41.6)
40–59	300 (48.8)
60–79	50 (8.1)
80+	2 (0.3)
Median BMI (kg/m^2) (IQR)	20 (18–22)
Median wt (kg) (IQR)	60 (53–65)
Median ht (cm) (IQR)	172 (166–177)
No. of participants with HIV-positive infection status/total no. of participants (%)	35/470 (7.4)
No. of participants with history of treated TB/total no. of participants (%)	233/618 (37.7)

[a]Human immunodeficiency virus (HIV)-positive infection status refers to the number of laboratory-confirmed HIV-positive participants out of the total number of participants tested. IQR, interquartile range; BMI, body mass index.

[b]The total number for each row may be lower than the number included in the study as participants could opt out of responding to questionnaire items.

were rifampin monoresistant, 185 (59.7%) were MDR-TB, 78 (25.2%) were pre-XDR-TB, and 2 (0.6%) had other resistance patterns.

The performance of the XDR-LFC assay using pDST as a reference standard is shown in Table 5. The two drugs with the highest sensitivities by the XDR-LFC were for isoniazid and rifampin, at 100% and 99.2%, respectively, and the lowest was for capreomycin, at 54.3%. Specificities were 100% for isoniazid, capreomycin, and amikacin; the lowest specificity was 84.1% for kanamycin. The overall accuracy of the assay ranged from 85.5% for kanamycin to 100% for isoniazid. The use of a composite phenotypic-genotypic reference standard was proposed previously (22), and the performance of the XDR-LFC against such a composite phenotypic-genotypic reference can be found in Table S4 in the supplemental material.

The 2021 WHO *Catalogue of Mutations in Mycobacterium tuberculosis Complex and Their Association with Drug Resistance* (7) lists the *eis* promoter −14t mutation as resistance conferring for amikacin. The XDR-LFC was designed prior to that publication and does not report this mutation as resistance conferring for amikacin; however, its reporting algorithm could be modified to do so. If the *eis* promoter −14t mutation were to be reported as resistance conferring for amikacin, the sensitivity of the XDR-LFC for amikacin would increase to 83.3% (95% confidence interval [CI], 64.2 to 93.3%), while the specificity would decrease to 97.2% (95% CI, 94.6 to 98.6%).

Evaluation of XDR-LFC performance using whole-genome sequencing. A total of 272 samples had valid XDR-LFC, pDST, and WGS results available. Excluding samples with indeterminate XDR-LFC results or missing WGS data due to inadequate gene coverage or missing positions at gene loci of interest, WGS isoniazid resistance results were available for 258 samples, rifampin resistance results were available for 266 samples, fluoroquinolone resistance results were available for 267 samples, kanamycin resistance results were available for 266 samples, and capreomycin and amikacin resistance results were available for 271 samples.

While the XDR-LFC was not designed to report specific mutations for clinical use, these data were available in the raw data for research evaluation. For samples with available XDR-LFC and WGS data, the XDR-LFC-reported mutations were compared to mutations detected by WGS and evaluated for concordance; these are shown stratified by pDST and XDR-LFC subcategories in Table 6. Results were considered concordant for a drug if the XDR-LFC reported a resistance-associated mutation(s) that was also identified by WGS. Results were considered discordant for a drug if the XDR-LFC

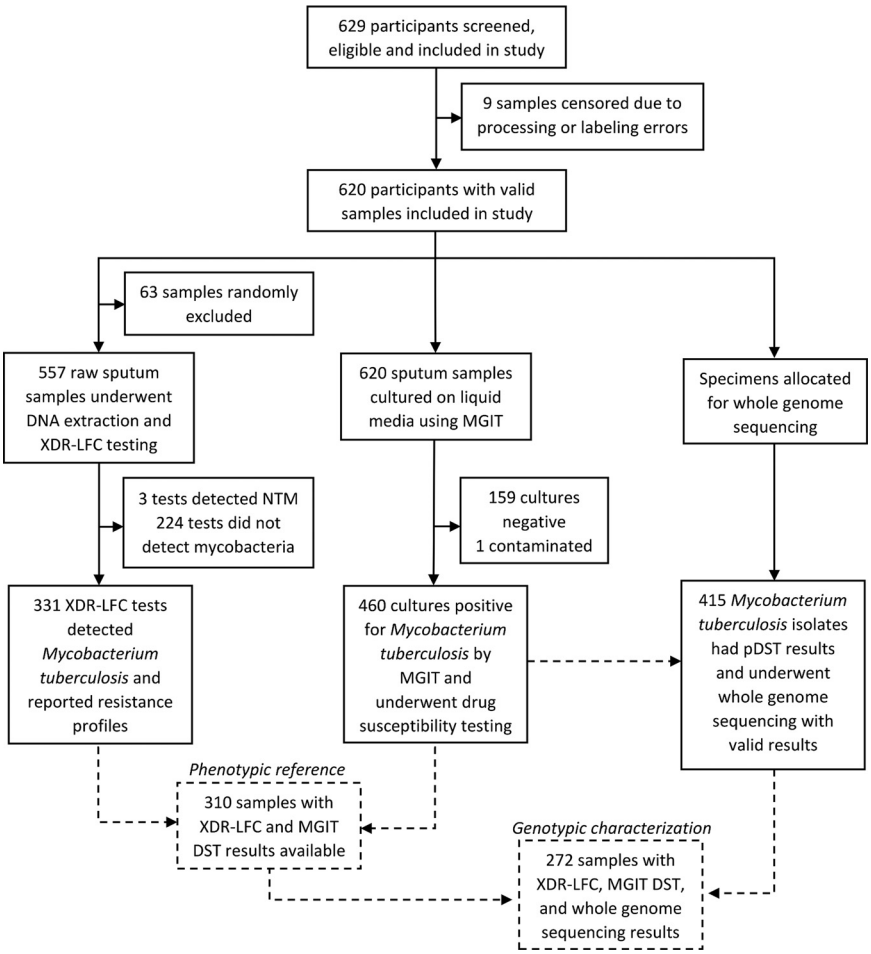

FIG 1 Study participant selection and sample processing flowchart.

reported a mutation(s) that was not identified by WGS or if WGS identified a resistance-conferring mutation(s) that was not detected by the XDR-LFC. In some instances, the resistance-conferring mutation(s) reported by WGS was not in the XDR-LFC assay's list of targets and was counted as a discordant result. The XDR-LFC and WGS results were concordant for 254 (98.4%) of 258 samples for isoniazid, 256 (96.2%) of 266 samples for rifampin, 253 (94.4%) of 268 samples for fluoroquinolones, 258 (97.0%) of 266 samples for kanamycin, 261 (96.3%) of 271 samples for capreomycin, and 267 (98.5%) of 271 samples for amikacin. However, when samples that had resistance-conferring mutations detected by WGS that were not in the XDR-LFC assay's list of gene targets were excluded, the numbers of XDR-LFC- and WGS-concordant results increased to 254 (98.8%) of 257 samples for isoniazid, 256 (98.8%) of 259 samples for rifampin, 253 (95.8%) of 264 samples for fluoroquinolones, 258 (97.7%) of 264 samples for kanamycin, 261 (99.2%) of 263 samples for capreomycin, and 267 (99.3%) of 269 samples for amikacin. Details of these discordant XDR-LFC and WGS results, with the associated mutation results, can be found in Table S2 in the supplemental material.

Incongruent resistance results between the XDR-LFC and pDST were also assessed using WGS data. No XDR-LFC and pDST results were incongruent for isoniazid. For rifampin, of the 2 samples that were rifampin resistant by pDST yet had no resistance-conferring mutations detected by the XDR-LFC, in both instances, WGS identified *rpoB* mutations (D435F and S450F) that were not included in the XDR-LFC target list (i.e., the XDR-LFC could not have detected them). For the one sample that was rifampin susceptible by pDST yet had a resistance-conferring mutation detected by the XDR-LFC, WGS

TABLE 3 XDR-LFC resistance results and *Mycobacterium tuberculosis* DNA extracted by sputum AFB smear status[a]

Drug(s) and XDR-LFC result	No. of isolates with XDR-LFC result by smear status			Median concn of *M. tuberculosis* DNA extracted by smear status (pg) (IQR)[b]		
	Negative	Scanty	Positive	Negative	Scanty	Positive
INH						
Susceptible	6	8	23	**1.14** (0.29–1.85)	0.55 (0.20–5.36)	**8.12** (1.45–42.10)
Resistant	62	31	187	0.52 (0.19–1.22)	0.93 (0.53–1.59)	**7.85** (2.06–40.40)
Indeterminate	4	3	7	0.52 (0.41–0.87)	0.34 (0.10–2.61)	**5.12** (4.00–132.00)
RIF						
Susceptible	9	8	34	0.48 (0.29–1.79)	0.55 (0.20–5.36)	**6.62** (1.66–42.10)
Resistant	63	34	182	0.55 (0.19–1.22)	0.91 (0.38–1.59)	**7.80** (2.06–31.60)
Indeterminate	0	0	1	N/A	N/A	**399.00** (399.00–399.00)
FQ						
Susceptible	59	33	166	0.50 (0.20–1.47)	0.69 (0.30–1.57)	**7.55** (1.80–31.60)
Resistant	13	9	50	0.54 (0.29–1.11)	**1.18** (0.86–2.61)	**11.05** (2.32–56.40)
Indeterminate	0	0	1	N/A	N/A	0.00 (0.00–0.00)
KAN						
Susceptible	40	16	109	0.50 (0.29–1.72)	0.61 (0.20–2.26)	**8.12** (1.45–42.90)
Resistant	28	25	105	0.57 (0.24–1.21)	0.93 (0.58–1.59)	**7.35** (2.32–28.40)
Indeterminate	4	1	3	0.05 (0.01–0.12)	0.14 (0.14–0.14)	**56.40** (0.00–150.00)
CAP and AMK						
Susceptible	67	39	205	0.50 (0.20–1.48)	0.86 (0.30–1.66)	**7.76** (1.84–40.40)
Resistant	4	3	12	0.81 (0.55–1.13)	**1.03** (0.58–2.61)	**20.50** (3.21–65.65)
Indeterminate	1	0	0	0.05 (0.05–0.05)	N/A	N/A
Total						
M. tuberculosis detected	72	42	217	0.52 (0.21–1.34)	0.87 (0.34–1.66)	**7.85** (1.86–41.90)
M. tuberculosis not detected	204	12	10	0.00 (0.00–0.00)	0.00 (0.00–0.06)	0.04 (0.00–0.06)
Total	276	54	227	0.00 (0.00–0.15)	0.58 (0.13–1.45)	**6.69** (1.45–31.60)

[a]Results are shown for the 557 samples that underwent testing with the XDR-LFC assay (Akonni Biosystems XDR-TB TruArray and lateral-flow-cell assay), excluding 9 samples deemed to have sample processing errors. "Positive" indicates a smear grade of 1+ or higher. IQR, interquartile range; INH, isoniazid; RIF, rifampin; FQ, fluoroquinolones (moxifloxacin and levofloxacin combined); CAP, capreomycin; AMK, amikacin.

[b]Median detected *M. tuberculosis* DNA values above 1 pg per reaction mixture are shown in boldface type. N/A, not applicable. See Table S3 in the supplemental material for additional details.

identified the same *rpoB* mutation as the one identified by the XDR-LFC (D435Y). Of the 12 samples that were fluoroquinolone resistant by pDST yet had no resistance-conferring mutations detected by the XDR-LFC, 4 samples had resistance-associated *gyrB* mutations (T500N, T500P, and 2 samples with D461N) identified by WGS that were not targeted by the XDR-LFC. In the remaining 8 samples that were resistant to fluoroquinolones by pDST, WGS identified no resistance-conferring mutations in 3 samples, and WGS was not done for 1 sample. Of the 2 samples that were fluoroquinolone susceptible by pDST yet had resistance-conferring mutations detected by the XDR-LFC, both had *gyrA* mutations (D94A and D94G) identified by WGS, of which one mutation was discordant from the XDR-LFC-reported suspected mutation.

Incongruent resistance results for the SLIDs between the XDR-LFC and pDST were also evaluated using WGS data. Of the 19 samples that were kanamycin resistant by pDST yet had no resistance-conferring mutations detected by the XDR-LFC, WGS did not detect any resistance-conferring mutations in 14 samples, WGS detected resistance-conferring mutations in 2 samples (*eis* promoter −12t in one sample and *rrs* C1402A and *rrs* G1484T in the other), and WGS was not done for 3 samples. Of the 25 samples that were kanamycin susceptible by pDST yet had resistance-conferring mutations detected by the XDR-LFC, WGS identified identical mutations in 24 samples, and WGS was not done for 1 sample. Of the 16 samples that were capreomycin resistant by pDST yet had no resistance-conferring mutations detected by the XDR-LFC, WGS

TABLE 4 Phenotypic resistance patterns of the 310 *Mycobacterium tuberculosis* culture-positive samples with XDR-LFC resistance profiles available[a]

	No. of isolates with resistance phenotype (%)[b]	
Drug(s)	Resistant	Susceptible
Isoniazid	272 (88)	38 (12)
Rifampin	263 (85)	47 (15)
Fluoroquinolones	79 (25)	231 (76)
Kanamycin	150 (48)	160 (52)
Capreomycin	35 (11)	275 (89)
Amikacin	24 (8)	286 (92)

[a]Phenotypic resistance results by MGIT for samples that were culture positive and for which XDR-LFC results were available are shown. Fluoroquinolone resistance refers to either levofloxacin or moxifloxacin resistance.
[b]Liquid MGIT DST critical concentrations for each drug were as follows: 0.1 μg/mL for isoniazid, 1.0 μg/mL for rifampin, 0.25 μg/mL for moxifloxacin and 1.5 μg/mL for levofloxacin (combined to represent fluoroquinolones), 2.5 μg/mL for kanamycin, 2.5 μg/mL for capreomycin, and 1.0 μg/mL for amikacin.

identified *tlyA* mutations not included in the XDR-LFC in 6 samples, WGS did not identify any resistance-conferring mutations in 9 samples, and 1 sample did not have WGS data available due to poor gene coverage. There were no samples that were capreomycin susceptible by DST with resistance-conferring mutations reported by the XDR-LFC. Of the 5 samples that were amikacin resistant by pDST yet had no resistance-conferring mutations detected by the XDR-LFC, WGS did not identify any resistance-conferring mutations in 4 samples, and WGS was not done for 1 sample. There were no samples that were amikacin susceptible by DST with resistance-conferring mutations reported by the XDR-LFC.

DISCUSSION

In this study, we evaluated a novel Akonni Biosystems XDR-TB TruArray and lateral-flow-cell assay using prospectively collected clinical samples. The proportion of male

TABLE 5 Summary of XDR-LFC results against MGIT phenotypic drug susceptibility testing[a]

		No. of isolates with MGIT pDST result					
Drug(s)	XDR-LFC result	Resistant	Susceptible	Culture negative	% sensitivity (95% CI)	% specificity (95% CI)	% accuracy
Isoniazid	Resistant	263	0	3	100 (98.6–100.0)	100 (89.9–100.0)	100
	Susceptible	0	34	17			
	Indeterminate	9	4	1			
Rifampin	Resistant	260	1	3	99.2 (97.3–99.8)	97.9 (88.9–99.6)	99.0
	Susceptible	2	46	18			
	Indeterminate	1	0	0			
Fluoroquinolones	Resistant	67	2	3	84.8 (75.3–91.1)	99.1 (96.9–99.8)	95.5
	Susceptible	12	228	18			
	Indeterminate	0	1	0			
Kanamycin	Resistant	127	25	6	87.0 (80.6–91.5)	84.1 (77.6–89.0)	85.5
	Susceptible	19	132	14			
	Indeterminate	4	3	1			
Capreomycin	Resistant	19	0	0	54.3 (38.2–69.5)	100 (98.6–100.0)	94.8
	Susceptible	16	275	20			
	Indeterminate	0	0	1			
Amikacin	Resistant	19	0	0	79.2 (59.5–90.8)	100 (98.7–100.0)	98.4
	Susceptible	5	286	20			
	Indeterminate	0	0	1			

[a]Fluoroquinolone resistance refers to either levofloxacin or moxifloxacin resistance. A "resistant" XDR-LFC assay (Akonni Biosystems XDR-TB TruArray and lateral-flow-cell assay) result indicates the detection of one or more resistance-conferring mutations, and a "susceptible" result indicates no detection of resistance-conferring mutations.

TABLE 6 XDR-LFC-suspected mutations compared to mutations detected by whole-genome sequencing arranged by phenotypic drug susceptibility testing result[a]

Drug(s)	MGIT pDST result	XDR-LFC result	No. of isolates with result by WGS (%)		
			XDR-LFC and WGS concordant	XDR-LFC and WGS discordant[b]	No WGS data[c]
Isoniazid	R	R	221 (98)	4 (2)	38
		S	0	0	0
	S	R	0	0	0
		S	33 (100)	0 (0)	1
Rifampin	R	R	214 (98)	5 (3)	41
		S	0 (0)	2 (100)	0
	S	R	1 (100)	0 (0)	0
		S	41 (93)	3 (7)	2
Fluoroquinolones	R	R	53 (93)	4 (7)	10
		S	3 (27)	8 (73)	1
	S	R	1 (50)	1 (50)	0
		S	196 (99)	1 (1)	31
Kanamycin	R	R	104 (96)	4 (4)	19
		S	14 (88)	2 (13)	3
	S	R	24 (100)	0 (0)	1
		S	116 (98)	2 (2)	14
Capreomycin	R	R	15 (94)	1 (6)	3
		S	9 (60)	6 (40)	1
	S	R	0	0	0
		S	237 (99)	3 (1)	35
Amikacin	R	R	15 (94)	1 (6)	3
		S	4 (100)	0 (0)	1
	S	R	0	0	0
		S	248 (99)	3 (1)	35

[a]Samples with no *M. tuberculosis* detected or indeterminate results by the XDR-LFC assay (Akonni Biosystems XDR-TB TruArray and lateral-flow-cell assay) are not shown. Percentages represent the proportions of concordant and discordant results and may not sum to 100 due to rounding. Fluoroquinolones include moxifloxacin and levofloxacin combined. pDST, phenotypic drug susceptibility testing; R, resistant; S, susceptible.

[b]Any discordance in one or more genes associated with the listed drug. Note that there are isolates where one gene is concordant but another is discordant, and in these cases, the isolates are referred to here as discordant. Further details can be found in Table S2 in the supplemental material.

[c]Whole-genome sequencing data were unavailable for isolates for which sequencing was not done or for which quality control parameters were not met.

participants seen in our study (79.5%) was noted to be higher than the global average (1); however, an increased risk of MDR/RR-TB among men in former Soviet Union countries, including the Republic of Moldova, has been described previously (23). The human immunodeficiency virus (HIV) positivity rate of 7.4% was in line with WHO and United Nations (UN) published rates and trends of TB and HIV coinfection (24, 25).

Among participants with MGIT culture-positive samples, the XDR-LFC detected *M. tuberculosis* in 96.4% of samples with AFB smear grades of 1+ or higher and in 48.7% of samples with negative or scanty smears. This pattern of higher rates of *M. tuberculosis* detection in individuals with AFB smear-positive sputum is seen with other assays, including Xpert MTB/RIF (26–29). In addition, the XDR-LFC was able to detect *M. tuberculosis* in 15.0% of the sputum samples from which *M. tuberculosis* did not grow in culture. The inability of the XDR-LFC assay to detect *M. tuberculosis* that was identified by other means, such as MGIT or Lowenstein-Jensen culture, was noted to occur with sputum samples that had insufficient concentrations of input DNA (~1 pg), with possible causes being related to the DNA extraction and purification efficiencies or low bacillary loads present in the decontaminated sputum samples.

Although most screened participants were able to provide the requested sputum volume, in addition to potentially decreasing the number of participants recruited to the study, it is also possible that the enrollment requirement for an 8.5-mL sputum volume favored the selection of more symptomatic participants with presumably higher bacillary loads.

The XDR-LFC provides profiling of *M. tuberculosis* resistance to four drug classes, and the WHO provides target product profiles (TPPs) for new tuberculosis drug susceptibility testing for isoniazid, rifampin, fluoroquinolones, and amikacin (30). The WHO TPP minimal requirement for sensitivity is >90% for isoniazid compared against pDST as a reference standard, and the optimal sensitivity is >95%. The WHO TPP minimal and optimal specificity requirements for isoniazid are ≥98% compared against pDST. The XDR-LFC isoniazid sensitivity and specificity were both 100%, exceeding the WHO TPP goals.

Similarly, the XDR-LFC sensitivity of 99.2% and specificity of 97.9% (98% when rounded to the nearest whole percentage) for rifampin exceeded the WHO TPP minimal and optimal sensitivity requirements of >95% and met the minimal and optimal specificity requirements of ≥98% compared against pDST. Nine samples had *rpoB* mutations that have been described as "low-MIC mutations" with associated MICs that fell below the WHO critical concentration for rifampin (31). When these mutations are excluded, the sensitivity for rifampin falls slightly to 96%, while the specificity increases to 100%. However, these mutations are known to clinically confer rifampin resistance, and current WHO guidelines designate these mutations resistance-conferring and clinically relevant mutations. Accordingly, these mutations were included and considered resistance conferring in this study. However, as the sole XDR-LFC "false-positive" result had a low-MIC mutation (*rpoB* D435Y) detected, the isolate may have been clinically resistant despite its classification as susceptible by the pDST reference standard (32).

The XDR-LFC sensitivity of 84.8% for fluoroquinolones fell short of the WHO TPP minimal and optimal requirements for sensitivity, which are >90% and >95%, respectively, against pDST as a reference. Of the 12 XDR-LFC samples with "false-negative" results against pDST, 4 samples had *gyrA* mutations that are included in the XDR-LFC but were not detected. An additional 4 samples had *gyrB* mutations identified by WGS that were not included in the XDR-LFC assay. In the 2021 WHO *Catalogue of Mutations in Mycobacterium tuberculosis Complex and Their Association with Drug Resistance* (7), using the currently recognized resistance-conferring mutations for fluoroquinolones, the combined sensitivity for levofloxacin was 84.4%, and that for moxifloxacin was 87.7%; these values fall within ranges similar to the sensitivities observed in our study. Furthermore, while the TBProfiler list of mutations differs from the WHO catalog of mutations for fluoroquinolones, and the evaluation of WGS using TBProfiler against pDST was not a primary objective of this study, if WGS were to be used for the detection of fluoroquinolone resistance based on the TBProfiler list of mutations, the sensitivity would have been 88.6% (95% CI, 79.8 to 93.9%), which also falls below the WHO TPP target. This suggests that the limitations in XDR-LFC sensitivity for fluoroquinolone resistance detection in this study were driven more by the lack of resistance-conferring mutations in *gyrA* in the study isolate population than by assay detection errors. In contrast, the XDR-LFC specificity of 99.1% for fluoroquinolones exceeded the WHO TPP target of ≥98%.

The XDR-LFC amikacin sensitivity of 79.2% against pDST fell marginally below the WHO TTP minimal requirement for sensitivity of ≥80%, although our study was limited by the low number of amikacin-resistant samples included, resulting in a wide confidence interval (95% CI, 59.5 to 90.8%) for sensitivity. The specificity of the XDR-LFC against pDST was 100% for amikacin and exceeded the WHO TPP target of ≥98%. The WHO TPP does not have targets for kanamycin or capreomycin as the use of these agents is no longer recommended by the WHO. The XDR-LFC sensitivity of 87.0% and specificity of 84.1% for kanamycin against pDST were lower than the performances reported for the Xpert MTB/XDR assay (33). However, when WGS using the TBProfiler mutation list was compared against pDST, the sensitivity was found to be 87.4% (95% CI, 80.5 to 92.1%), and the specificity was 80.7% (95% CI, 73.5 to 86.3%), suggesting

limitations inherent in using currently known resistance-conferring mutations to predict phenotypic drug susceptibility among the samples included in this study. While the specificity of the XDR-LFC for capreomycin was 100%, the sensitivity was 54.3% against pDST. Using WGS against pDST, the current theoretical sensitivity achievable using genotyping was 65.6% (95%, CI 48.3 to 79.6%) for the samples studied. Thus, if more reliably predictive resistance mutations for aminoglycosides are identified in the future, the inclusion of these new targets in the XDR-LFC assay would likely improve the performance of the platform.

A limitation of the XDR-LFC is that it does not distinguish between high-level and low-level resistance conferred by mutations, which may be clinically relevant for some individuals. In the instance of one isolate (BP2-4088), the XDR-LFC reported fluoroquinolone resistance based on the detection of a D94A mutation, while WGS identified a D94G mutation. While the D94G mutation confers high-level resistance precluding fluoroquinolone use, high-dose moxifloxacin might be considered for the lower-level resistance conferred by the D94A mutation. Furthermore, while a related assay for influenza detection was able to detect variants at a lower limit of quantitation of 1 to 5% within a mixed population (34), XDR-LFC characteristics, including the limit of detection for low-frequency mutations and performance on samples with heteroresistance, have not been characterized in a similar fashion.

It is also important to recognize that testing and treatment guidelines have changed rapidly over the past few years. The XDR-LFC does not detect bedaquiline or linezolid resistance and therefore is not designed to be used for the detection of XDR-TB based on the new 2021 WHO definition (4). Furthermore, critical drug concentrations at the time of study implementation were based on WHO recommendations from 2008 and 2012, and new WHO-recommended critical drug concentrations and MDR-TB treatment regimens have been published since this study was designed and conducted. Our study used a critical concentration of 1.5 μg/mL for levofloxacin, and the critical concentration for levofloxacin by MGIT was decreased from 2.0 μg/mL to 1.5 μg/mL in 2012 (17) and, subsequently, to 1.0 μg/mL in 2018 (35). Our study used a critical concentration of 1.0 μg/mL for rifampin, and the critical concentration for rifampin was lowered from 1.0 μg/mL to 0.5 μg/mL in 2021 (32). As TB resistance definitions, DST guidelines, and recommended treatment regimens change over time, rapid DST approaches will need to evolve concurrently. One strength of the XDR-LFC assay is that the platform remains flexible for probe target exchange or expansion to meet future requirements without the need for fundamental hardware or instrumentation changes. An additional strength of the XDR-LFC assay is that, in comparison to WHO-approved rapid molecular workflows such as GeneXpert MTB/RIF Ultra and MTB/XDR (Cepheid, Sunnyvale, CA, USA) and GenoType MTBDRplus and MTBDRsl (Bruker/Hain Lifescience, Nehren, Germany) that rely on a multistep protocol to achieve comparable drug resistance profiling, the XDR-LFC assay is designed to run in a single step using a single clinical sample.

Conclusions. The application of the XDR-LFC assay for clinically relevant resistance prediction in a prospective study population demonstrates the strengths of the assay in testing for MDR-TB, pre-XDR-TB, and SLID-resistant TB directly from sputum samples on a single platform and without sample splitting in a population at high risk for drug-resistant tuberculosis. The ability to use decontaminated sputum to rapidly test for the presence of *M. tuberculosis* and multiple resistance-conferring mutations across seven genes via a multiplexed approach provides time and resource advantages over traditional culture and phenotypic drug susceptibility testing. The performance of the assay met WHO TPP standards for new tuberculosis drug susceptibility testing for isoniazid and rifampin. While the assay's sensitivity for fluoroquinolone resistance detection did not meet the WHO TPP target, improving the assay with *gyrB* targets and testing a larger global cohort would likely enable the assay to reach TPP criteria. The specificities for both fluoroquinolones and amikacin met WHO TPP goals, while the sensitivity for amikacin was close to but did not meet the goal of $\geq$80%. Ranging from 95 to 99%, the high degree of concordance between the XDR-LFC and WGS results suggests that

the platform performed well in detecting resistance-conferring mutations when they were present. Furthermore, when considering only samples with mutations for which the XDR-LFC has targets, the concordance increased to 96 to 99%, indicating the potential for performance improvement if additional targets are added.

Overall, the XDR-LFC represents a promising new technology for the rapid detection of *M. tuberculosis* and drug resistance profiles from direct clinical samples. Rapid sputum-based testing for resistance to drugs, including bedaquiline, linezolid, pretomanid, and delamanid, among others, will be increasingly valuable with widespread use. However, when considered against current standards, the implementation of the current sputum-based XDR-LFC assay in clinical and public health applications stands to improve rapid resistance detection capabilities and corresponding treatment decision-making. This diagnostic assay is particularly promising in communities with a high prevalence of TB with isoniazid, rifampin, or fluoroquinolone resistance as well as in clinical scenarios in which SLIDs are being used or considered.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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