Diagnostic Accuracy and Utility of FluoroType MTBDR, a New Molecular Assay for Multidrug-Resistant Tuberculosis

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ABSTRACT Most cases of multidrug-resistant (MDR) tuberculosis (TB) are never di-agnosed (328,300 of the ~490,000 cases in 2016 were missed). The Xpert MTB/RIF assay detects resistance only to rifampin, despite ~20% of rifampin-resistant cases being susceptible to isoniazid (a critical first-line drug). Consequently, many coun-tries require further testing with the GenoType MTBDR*plus* assay. However, MTBDR-*plus* is not recommended for use on smear-negative specimens, and thus, many specimens require culture-based drug susceptibility testing. Furthermore, MTBDR*plus* requires specialized expertise, lengthy hands-on time, and significant laboratory in-frastructure and interpretation is not automated. To address these gaps, we evalu-ated the accuracy of the FluoroType MTBDR (FluoroType) assay. Sputa from 244 smear-positive and 204 smear-negative patients with presumptive TB (Xpert MTB positive, $n = 343$) were tested. Culture and MTBDR*plus* on isolates served as refer-ence standards (for active TB and MDR-TB, respectively). Sanger sequencing and MT-BDR*plus*, both of which were performed on sputa, were used to resolve discrepan-cies. The sensitivity of FluoroType for the detection of *M. tuberculosis* complex was 98% (95% confidence interval [CI], 95 to 99%) and 92% (95% CI, 84 to 96%) for smear-positive and smear-negative specimens, respectively (232/237 versus 90/98 speci-mens; $P < 0.009$). The sensitivity and specificity for smear-negative specimens were 100% and 97%, respectively, for rifampin resistance; 100% and 98%, respectively, for isoniazid resistance; and 100% and 100%, respectively, for MDR-TB. FluoroType iden-tified 98%, 97%, and 97% of the *rpoB*, *katG*, and *inhA* promoter mutations, respec-tively. FluoroType has excellent sensitivity with sputa equivalent to that of MTBDR-*plus* with the isolates and can provide rapid drug susceptibility testing for rifampin and isoniazid. In addition, the capacity of FluoroType to simultaneously identify vir-tually all mutations in the *rpoB*, *katG*, and *inhA* promoter may be useful for individu-alized treatment regimens.

KEYWORDS FluoroType MTBDR, tuberculosis, drug resistance, diagnostics, molecular,
Mycobacterium tuberculosis, isoniazid, rifampin

The underdiagnosis of tuberculosis (TB) remains a major hurdle for the eradication of the disease. The World Health Organization (WHO) recommends that all individ-uals presenting with symptoms or signs of TB be tested with either the Xpert MTB/RIF (Xpert) assay or its successor (Ultra). Individuals diagnosed with rifampin-resistant TB are then initiated on an anti-multidrug-resistant (MDR) TB treatment regimen (1). It is therefore recommended that treatment subsequently be optimized following confir-matory testing for resistance to rifampin, isoniazid, and second-line anti-TB drugs (1).

However, confirmatory testing is frequently not done in many settings and rifampin monoresistance thereby fails to be recognized (~20%) (2, 3).

Only one widely used commercial assay available for MDR-TB exists: GenoType MTBDR*plus* (MTBDR*plus*; Hain Lifescience GmbH, Nehren, Germany). WHO has endorsed MTBDR*plus*, but it is recommended for use only on smear-positive specimens. Smear-negative patients, who are common in high-HIV infection-burden settings, hence first require culture-based drug susceptibility testing (DST), which causes significant delays in diagnosis and initiation of treatment. MTBDR*plus* is a line probe assay that produces amplicons in an open-tube format and therefore requires an experienced operator, a minimum of three separate rooms, and strict adherence to standard operating procedures to minimize the risk of amplicon cross contamination. These assays also require experienced readers for the interpretation of the hybridized bands, although this can be semiautomated at added expense.

The new assay was invented and constructed at Brandeis University and commercialized as the FluoroType MTBDR (FluoroType) assay by Hain Lifescience GmbH. It is designed as a qualitative *in vitro* test for the automated detection of the *Mycobacterium tuberculosis* complex and resistance to rifampin and isoniazid directly from sputum specimens. Detection of rifampin resistance is enabled by identification of mutations in the rifampin resistance-determining region of the *rpoB* gene. Isoniazid resistance is identified through mutations in *katG* and the *inhA* promoter region.

FluoroType uses a nonsymmetric PCR (previously described as a linear after the exponential [LATE] PCR), together with sets of lights-on/lights-off probes (4, 5). The design and construction of this assay have been discussed in detail (our unpublished data). Briefly the assay works as follows: primers amplify separate amplicons for the *rpoB*, *inhA* promoter, and *katG* gene targets plus an internal control. The single-stranded products are detected at the endpoint by melt curve analysis of the hybridized sets of lights-on/lights-off probes (5). The collective fluorescent signals from all probes comprise a temperature-dependent fluorescent signature in a single color. Each fluorescent signature is automatically interpreted by the Fluoro-Software IVD to reliably distinguish and classify sequences, even when they differ by a single nucleotide.

The aim of this study was to evaluate the diagnostic performance of FluoroType using DNA extracted from sputum specimens and to compare the results to those obtained with MTBDR*plus*.

MATERIALS AND METHODS

Clinical specimens. A total of 448 sputum specimens were collected from Xpert MTB/RIF-positive and -negative patients at the National Health Laboratory Services, Green Point, Cape Town, South Africa. Sputum specimens were decontaminated with NaOH–*N*-acetyl-L-cysteine (final concentration, 1%) and resuspended in 2 ml phosphate-buffered saline. Thereafter, 50 μl was subjected to auramine O smear microscopy, and 0.5 ml used to inoculate an MGIT culture. DNA was extracted only from Xpert MTB/RIF-positive sputum sediments and the corresponding positive MGIT cultures using a GenoLyse kit (Hain Lifescience GmbH, Nehren, Germany), and drug susceptibility testing was done on both using the MTBDR*plus* assay prior to storage of the DNA extracts at −20°C. The residual sediments were stored at 4°C for a maximum of 48 h prior to transport and storage at Stellenbosch University at −20°C. A unique study number was assigned to each specimen, and patient identifiers were removed. A waiver of consent has been granted for this study by the Stellenbosch University Health Research Ethics Committee (reference no. N12/01/001).

DNA extraction. DNA was extracted from the remaining Xpert MTB/RIF-positive and -negative sputum sediments using a FluoroLyse kit (Hain Lifescience GmbH) according to the manufacturer's instructions. Briefly, a 500-μl aliquot of the residual resuspended sputum sediment was centrifuged for 15 min at 10,000 × *g* to pellet the bacilli. The resulting pellet was resuspended in 100 μl lysis buffer (F-LYS) (mixed with 2 μl of the amplification control [β-IC]) by vortexing. The mixture was then incubated for 5 min at 95°C. Subsequently, 100 μl of the neutralization buffer (F-NB) was added, and the mixture was vortexed for 5 s and then centrifuged for 5 min at full speed (14,000 × *g*). The supernatant was then transferred to a new tube and stored at −20°C until further use. A tube containing no clinical material was included during the extraction protocol as a negative control.

FluoroType MTBDR assay. FluoroType tests were done using the FluoroCycler96 instrument. PCR mixes were freshly prepared by combining 6 μl amplification mix A (AM-A) and 14 μl amplification mix B (AM-B). Thereafter, 20 μl of the FluoroLyse-extracted DNA was added to the PCR mix. Controls included 20 μl of the negative control prepared during the DNA extraction process or 20 μl of the positive control
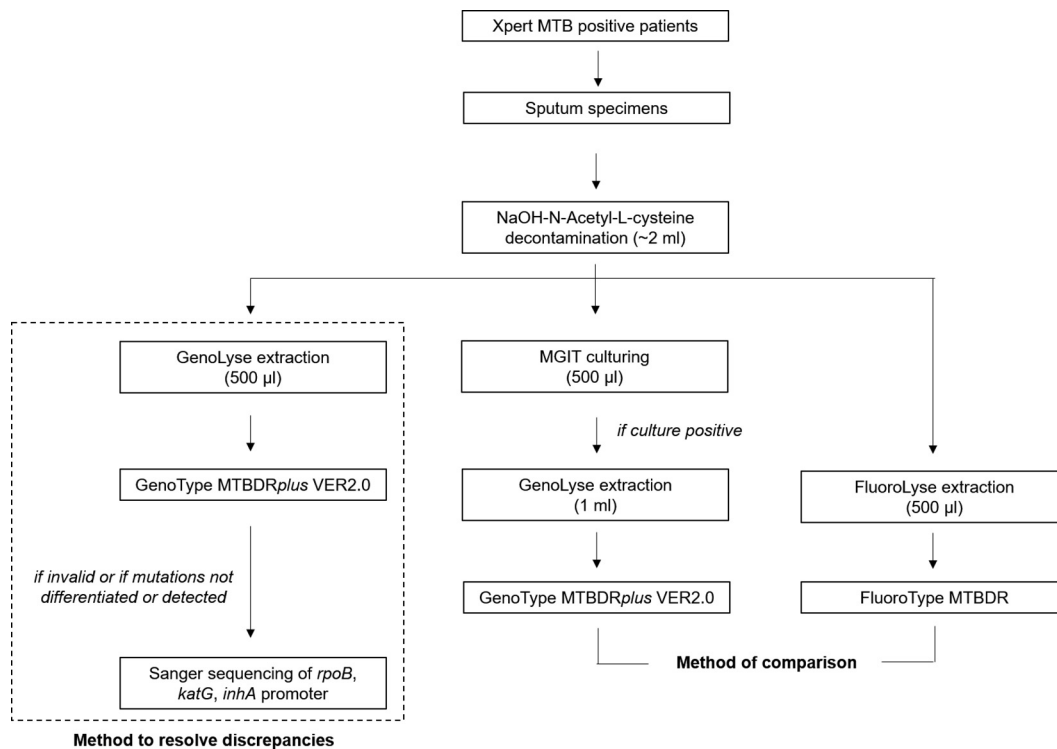
FIG 1 Description of methods used for comparison and discrepancy analysis for the detection of resistance to rifampin and isoniazid using the FluoroType MTBDR assay.

(β-C+). The PCR mixes were immediately loaded into the FluoroCycler96 instrument to avoid photo-bleaching.

The FluoroType analyzer software validates the positive and negative controls, whether *M. tuberculosis* complex DNA is identified through the presence of a positive signature for *rpoB*, and what the specific mutations for all three genes are. It also reports unknown signatures (but different from the wild type) as "MUT" (undifferentiated mutation) and indeterminate results (the software cannot differentiate between the wild type and a mutant) as "IND." In cases in which the positive, negative, or amplification control failed, "invalid" is reported by the software. Heteroresistance and/or mixed infections were counted as correctly identified if the underlying mutation was detected.

Analysis. For the detection of the *M. tuberculosis* complex, the FluoroType result was compared to the presence or absence of the TUB band from the MTBDR*plus* result obtained for the corresponding cultured isolates. For the detection of rifampin and isoniazid resistance, the FluoroType results were compared to the MTBDR*plus* hybridization patterns of the probes corresponding to the *rpoB* and *katG* genes and the *inhA* promoter region obtained from the corresponding cultured isolates (as not all samples have a valid MTBDR*plus* result from a specimen) (Fig. 1). For discrepant results, MTBDR*plus* results for DNA extracted from the sputum specimens with the GenoLyse kit were used as the reference. In cases in which no valid MTBDR*plus* result was obtained from sputum or MTBDR*plus* failed to differentiate or detect specific mutations, Sanger sequencing was also used as a reference method. Targeted Sanger sequencing for the resistance-determining regions of *rpoB*, *katG*, and the *inhA* promoter was done as previously described (6). In case of invalid results, the FluoroType assay was repeated and the second result was used for the final analysis.

Statistical analysis (two-sample test of proportions) was done using Stata statistical software, release 14.0 (StataCorp. 2015; StataCorp LP, College Station, TX).

RESULTS

A total of 448 sputum specimens were included in the study; 343 of these were collected from patients diagnosed as Xpert positive, while 105 sputum specimens were collected from patients diagnosed as Xpert negative (smear and culture negative). The 343 sputum specimens from Xpert-positive patients comprised 244 sputum smear-positive and 99 sputum smear-negative specimens (Fig. 2). The Xpert-positive samples comprised 132 (38%) rifampin- and isoniazid-sensitive specimens, 35 (10%) rifampin-monoresistant specimens, 13 (4%) isoniazid-monoresistant specimens, and 163 (48%) MDR specimens, as characterized by MTBDR*plus* and sequencing.
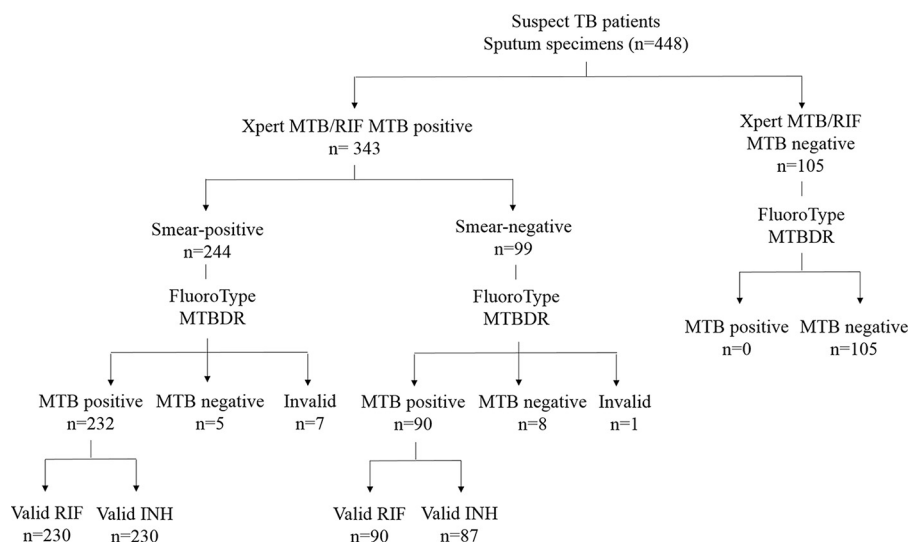
FIG 2 Description of specimens collected for the study broken down according to smear status, detection of the *M. tuberculosis* complex using FluoroType MTBDR, and determination of rifampin and isoniazid susceptibility using FluoroType MTBDR. Definitions of abbreviations: RIF, rifampin; INH, isoniazid; MTB, *M. tuberculosis* complex.

Detection of *M. tuberculosis* complex using FluoroType MTBDR. The sensitivity of the FluoroType assay for the detection of *M. tuberculosis* was 97.9% in smear-positive specimens and 91.8% in smear-negative specimens (Table 1) when using the presence or absence of the TUB band from the MTBDR*plus* culture-based result. In the smear-positive sample set, 5/244 of the specimens were not detected as *M. tuberculosis* complex positive. For 10 specimens, invalid results were obtained. After repeat testing, seven specimens remained with invalid results. In the smear-negative sample set (which included both culture-positive and culture-negative specimens), false-negative results were scored for 8 specimens. For 11 specimens, an invalid result was obtained. After repeat testing, only 1 specimen had an invalid result. The inhibition rate (number of tests reported as "invalid" by the analyzer software) for FluoroType was 1.8% (8/448) for sputum specimens. Table 1 also shows the performance of MTBDR*plus* done on the same set of sputum specimens. No significant difference was observed between FluoroType and MTBDR*plus* for the performance on smear-positive specimens (97.8% [95% confidence interval {CI}, 95.2 to 99.3%; 232/237] versus 99.2% [95% CI, 96.8 to 99.9%; 242/244], $P = 0.238$) and smear-negative specimens (91.8% [95% CI, 84.1 to 96.2%; 90/98] versus 92.3% [95% CI, 85.5 to 96.9%; 92/99], $P = 0.775$).

Detection of rifampin resistance using FluoroType MTBDR. For the detection of rifampin resistance from smear-positive specimens, an initial sensitivity and specificity of 96.9% and 97%, respectively, were achieved when the FluoroType results were compared to the MTBDR*plus* results for the corresponding cultured isolates (Table 2). This was not significantly different from the performance of MTBDR*plus* on the same set of sputum specimens (Table 2). Three of 3 false-resistant and 3 of 4 false-sensitive results of FluoroType were confirmed by the MTBDR*plus* result or Sanger sequencing using the GenoLyse extract from the sputum specimens (Table 3). The remaining false-sensitive result by FluoroType was caused by heteroresistance, identified by Sanger sequencing (Table 3). Therefore, the final sensitivity and specificity of Fluoro-Type for the detection of rifampin resistance from smear-positive specimens were 99.2% and 100%, respectively (Table 4). Only 2/232 smear-positive specimens (0.86%) showed an indeterminate FluoroType result.

In the smear-negative sample set, an initial sensitivity and specificity of 100% and 92.3%, respectively, were achieved when the FluoroType rifampin susceptibility results were compared to the MTBDR*plus* results obtained from cultured isolates

TABLE 1 Performance of FluoroType MTBDR and Genotype MTBDRplus for detection of *M. tuberculosis* complex from smear-positive and smear-negative sputum specimens compared to culture and Genotype MTBDRplus results obtained from the corresponding cultured isolates[a]

Specimen	FluoroType MTBDR[b]							MTBDRplus[c]			
	Sensitivity		Specificity				No. of invalid tests	Sensitivity			No. of invalid tests
	% (95% CI)	No. of specimens positive/total no. of samples tested	% (95% CI)	No. of specimens positive/total no. tested	% PPV (95% CI)	% NPV (95% CI)		% (95% CI)	No. of specimens positive/total no. tested	% PPV (95% CI)	
All (n = 448)	96.11 (93.3–97.8)	322/335	100 (95.6–100)	105/105	100 (98.5–100)	89 (81.6–93.8)	8	97 (94.5–98.8)	334/343	100 (98.6–100)	0
Sputum smear positive (n = 244)	97.9 (94.9–99.2)	232/237			100 (98.4–100)		7	99.2 (96.8–99.9)	242/244 (P = 0.24[d])	100 (98.0–100)	0
Sputum smear negative (n = 204)	91.8 (84.1–96.2)	90/98 (P = 0.009[e])	100 (96.6–100)	105/105	100 (96–100)	92.9 (86.5–96.9)	1	92.3 (85.5–96.9)	92/99 (P = 0.001[e], P = 0.78[d])	100 (95–100)	0

[a] Definitions of abbreviations: CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.
[b] Fluorotype MTBDR assay done on sputum specimens.
[c] Genotype MTBDRplus done on sputum specimens.
[d] P values comparing FluoroType MTBDR and Genotype MTBDRplus results within smear groups.
[e] Assay-specific P values comparing results for smear-positive and smear-negative specimens.

TABLE 2 Performance of FluoroType MTBDR and Genotype MTBDR*plus* for detection of rifampin and isoniazid resistance from smear-positive and smear-negative sputum specimens compared to GenoType MTBDR*plus* results obtained from DNA extracts from the corresponding cultured isolates[a]

	FluoroType MTBDR[b]						
	Rifampin						
		Sensitivity		Specificity			
Specimen	No. of indeterminate results	% (95% CI)	No. of specimens positive/total no. tested	% (95% CI)	No. of specimens positive/total no. tested	Positive LR (95% CI)	Negative LR (95% CI)
All (n = 322)		97.8 (94.1–99.3)	178/182	95.6 (90.4–98.2)	132/138	22.5 (10.3–49.2)	0.02 (0.009–0.06)
Sputum smear positive (n = 232)	2	96.9 (92.4–99.2)	127/131	97 (91.4–99.4)	96/99	32.3 (10.5–97.5)	0.03 (0.01–0.08)
Sputum smear negative (n = 90)	0	100 (93–100)	51/51 (P = 0.21[e])	92.3 (79.1–98.4)	36/39	13 (4.4–38.6)	0 (0–NaN)

[a]Definitions of abbreviations: CI, confidence intervals; LR, likelihood ratio; NaN, the calculation could not be performed because the values entered included one or more instances of zero.
[b]FluoroType MTBDR assay done on sputum specimens.
[c]Genotype MTBDR*plus* done on sputum specimens.
[d]P values comparing FluoroType MTBDR and Genotype MTBDR*plus* results within smear groups.
[e]Assay specific P values comparing results for smear-positive and smear-negative specimens.

(Table 2). This was not significantly different from the MTBDR*plus* result from the same set of sputum specimens (Table 2). Three false-resistant results of FluoroType were confirmed to be true resistant by the MTBDR*plus* assay done on the DNA extracted directly from the sputum specimens by use of the GenoLyse kit (Table 3). The final sensitivity and specificity of the FluoroType assay for the detection of rifampin resistance from smear-negative specimens were 100% and 97.3%, respectively, with no indeterminate results (Table 4). MTBDR*plus* done directly on the DNA extracted from the sputum specimens showed a higher indeterminate rate than FluoroType (6.5% [95% CI, 1.5 to 11.7%; 6/91] versus 0% [95% CI, 0 to 0%; 0/90], P = 0.01).

Detection of isoniazid resistance using FluoroType MTBDR. For the detection of isoniazid resistance from smear-positive specimens, an initial sensitivity and specificity of 98.3% and 97.3%, respectively, were achieved when the FluoroType result was compared to the MTBDR*plus* result obtained from the corresponding cultured isolates

TABLE 3 Resolution of discrepant FluoroType MTBDR rifampin and isoniazid resistance results using Sanger sequencing and Genotype MTBDR*plus* results obtained from DNA extracted from sputum specimens[a]

Antimicrobial	Smear result	Initial classification	MTBDR*plus* result[b]	FluoroType MTBDR result[c]	Method used to resolve discrepancy[d]	Final result	Reclassification
Rifampin	Positive	False resistant (n = 3)	WT	D516V	MTBDR*plus*	WT + D516V	True resistant
			WT	MUT	Sequencing	Q513P	True resistant
			WT	ins(514-515)	Sequencing	TTC ins(514-515)	True resistant
		False sensitive (n = 4)	S531L	WT	MTBDR*plus*	WT	True resistant
			WT + S531L	WT	MTBDR*plus*	WT	True resistant
			D516V	WT	MTBDR*plus*	WT	True resistant
			WT + D516V	WT	Sequencing	WT + D516V	False sensitive
	Negative	False resistant (n = 3)	WT	S531L	MTBDR*plus*	S531L	True resistant
			WT	MUT	Sequencing	WT + D516Y	True resistant
			WT	S531L	MTBDR*plus*	WT	False resistant
Isoniazid	Positive	False resistant (n = 3)	WT	S315T2	MTBDR*plus*	WT + S315T1	True resistant
			WT	S315T1	MTBDR*plus*	WT + S315T1	True resistant
			WT	MUT	MTBDR*plus*	WT	False resistant
		False sensitive (n = 2)	S315T2	WT	MTBDR*plus*	WT	True sensitive
			S315T1	WT	MTBDR*plus*	WT	True sensitive
	Negative	False resistant (n = 2)	WT	S315T1	MTBDR*plus*	S315T1	True resistant
			WT	C-15T	No valid result obtained		False resistant

[a]Definitions of abbreviations: WT, wild type; MUT, undifferentiated mutation; ins(514-515), insertion between codons 514 and 515.
[b]GenoType MTBDR*plus* result from DNA extracted from cultured isolate.
[c]FluoroType MTDR result from DNA extracted from sputum specimen.
[d]Discrepancies were resolved by using either the sequencing or GenoType MTBDR*plus* results from DNA extracted from sputum specimens.

TABLE 2 (Continued)

FluoroType MTBDR[b]						
Isoniazid						
	Sensitivity		**Specificity**			
No. of indeterminate results	% (95% CI)	No. of specimens positive/total no. tested	% (95% CI)	No. of specimens positive/total no. tested	Positive LR (95% CI)	Negative LR (95% CI)
	98.8 (95.1–99.8)	158/160	97 (92.6–98.9)	152/157	32 (13.5–75.8)	0.01 (0.003–0.05)
2	98.3 (94.1–99.8)	117/119	97.3 (92.3.–99.4)	108/111	36.4 (11.9–111.1)	0.02 (0.004–0.07)
3	100 (91.4–100)	41/41 ($P = 0.40^e$)	95.7 (85.2–99.5)	44/46	23.3 (5.93–89.2)	0 (0–NaN)

(Table 2). This was not significantly different from the results of MTBDR*plus* done on the same set of sputum specimens (Table 2). Using the MTBDR*plus* result from the sputum specimens, 2/3 false-resistant and 2/2 false-sensitive results of FluoroType were confirmed to be true resistant and true sensitive, respectively (Table 3). Therefore, the overall sensitivity and specificity of FluoroType for the detection of isoniazid resistance from smear-positive specimens were 100% and 99.1%, respectively (Table 4), with 2 (0.86%) specimens showing an indeterminate result.

In the smear-negative sample set, an initial sensitivity and specificity for the detection of isoniazid resistance of 100% and 95.7%, respectively, were achieved when the FluoroType results were compared to the results from the MTBDR*plus* assay done on the corresponding cultured isolates (Table 2). This was not significantly different from the MTBDR*plus* result done on sputum (Table 2). Two false-resistant results were detected by FluoroType, while 3/90 specimens (3.33%) showed indeterminate results (Table 2 and 3). One of the false-resistant results was confirmed to be true resistant by MTBDR*plus* assay analysis of DNA extracted from the sputum specimen by use of the GenoLyse kit, while for the second sample, no valid MTBDR*plus* or sequencing result could be obtained from the sputum specimen. Thus, the overall sensitivity and specificity of the FluoroType assay for the detection of isoniazid resistance in smear-negative specimens were 100% and 97.8%, respectively (Table 4).

Detection of MDR using the FluoroType MTBDR. When using Sanger sequencing and MTBDR*plus* (from specimens and the corresponding isolates) as the reference methods, FluoroType showed a sensitivity and a specificity of 99.1 and 100%, respectively, with smear-positive sputum specimens for the detection of MDR-TB (Table 5). With smear-negative specimens, FluoroType showed a sensitivity and a specificity of 100% for the detection of MDR-TB (Table 5).

Detection of specific resistance-associated mutations by the FluoroType MTBDR assay. The specific resistance-associated mutations in *rpoB*, *katG*, and the *inhA* promoter detected by the FluoroType assay were compared to the resistance muta-

TABLE 4 Corrected diagnostic performance of the FluoroType MTBDR assay for detection of rifampin and isoniazid resistance using Sanger sequencing and GenoType MTBDR*plus* as reference methods[a]

	Rifampin					
		Sensitivity		**Specificity**		
Specimen	No. of indeterminate results	% (95% CI)	No. of specimens positive/total no. tested	% (95% CI)	No. of specimens positive/total no. tested	Positive LR (95% CI)	Negative LR (95% CI)
All (*n* = 322)		99.5 (96.6–100)	183/184	99.3 (95.6–100)	135/136	135.3 (19.2–953.3)	0.01 (0.0008–0.04)
Sputum smear positive (*n* = 232)	2	99.2 (95.8–100)	130/131	100 (96.6–100)	99/99	Undefined (NaN–infinity)	0.01 (0.001–0.05)
Sputum smear negative (*n* = 90)	0	100 (93–100)	53/53	97.3 (85.8–99.9)	36/37	37.03 (5.4–255.8)	0 (0–NaN)

[a]Definitions of abbreviations: CI, confidence intervals; LR, likelihood ratio; NaN, the calculation could not be performed because the values entered included one or more instances of zero.

TABLE 2 (Continued)

Genotype MTBDRplus[c]						
Rifampin						
	Sensitivity		**Specificity**			
No. of indeterminate tests	**% (95% CI)**	**No. of specimens positive/total no. tested**	**% (95% CI)**	**No. of specimens positive/total no. tested**	**Positive LR (95% CI)**	**Negative LR (95% CI)**
	97.3 (93.5–99)	181/186	91.5 (85.3–95.3)	129/141	11.4 (6.7–19.6)	0.03 (0.01–0.07)
0	97.1 (92.2–99.1)	132/136 (P = 0.95[d])	95.3 (88.8–98.3)	101/106	20.58 (8.74–48.4)	0.03 (0.01–0.08)
6	98 (88–99.9)	49/50 (P = 0.73,[e] P = 0.31[d])	80 (62.5–90.9)	28/35	4.9 (2.5–9.5)	0.03 (0.003–0.18)

tions identified by MTBDRplus and Sanger sequencing (Tables 6 to 8). Since the FluoroType assay can output only one result per gene, heteroresistance and/or mixed infections were regarded as correctly interpreted if the software was able to identify the specific resistance-associated mutation. Overall, the FluoroType assay correctly identified 97.5% (314/322), 96.6% (311/322), and 96.6% (311/322) of the rpoB, katG, and inhA promoter mutations, respectively (Tables 6 to 8).

DISCUSSION

This was the first study to evaluate the diagnostic performance of the FluoroType assay with smear-positive and -negative sputum specimens. Our key findings are as follows: (i) FluoroType showed increased sensitivity for the detection of the M. tuberculosis complex in smear-negative sputum specimens, (ii) FluoroType was highly comparable to MTBDRplus for the detection of resistance to rifampin and isoniazid irrespective of smear gradation, (iii) FluoroType showed a high accuracy for the identification of mutations in rpoB, katG, and the inhA promoter, and (iv) FluoroType is a simple, rapid closed-tube molecular test that can provide operator-independent results. These findings suggest that FluoroType can be used as a replacement test for MTBDRplus. (v) Interpretation of FluoroType data is fully automated, thereby removing operator interpretation subjectivity.

FluoroType showed increased sensitivity (91.8%) for the detection of the M. tuberculosis complex in smear-negative sputum specimens compared to that previously shown for MTBDRplus (40 to 100%) (7–12). In this study, no significant difference in the performance of MTBDRplus (sensitivity, 92.3% [95% CI, 85.5 to 96.9; 92/99], P = 0.76) from that of FluoroType with smear-negative sputum specimens was observed. This is not unexpected, as all specimens tested were diagnosed as Xpert positive, and similar agreement between MTBDRplus and Xpert has been reported (13). We did not test the performance of FluoroType with Xpert TB-negative TB culture-positive specimens, as the specimen inclusion criteria were based on the current diagnostic algorithm used in

TABLE 4 (Continued)

Isoniazid						
	Sensitivity		**Specificity**			
No. of indeterminate results	**% (95% CI)**	**No. of specimens positive/total no. tested**	**% (95% CI)**	**No. of specimens positive/total no. tested**	**Positive LR (95% CI)**	**Negative LR (95% CI)**
	100 (97.1–100)	161/161	98.7 (95–99.8)	154/156	78 (19.7–309.1)	0 (0–NaN)
2	100 (97–100)	119/119	99.1 (95.1–100)	110/111	111.1 (15.8–781.1)	0 (0–NaN)
3	100 (91.6–100)	42/42	97.8 (88.2–99.9)	44/45	45.5 (6.48–312.5)	0 (0–NaN)

TABLE 2 (Continued)

Genotype MTBDR*plus*[c]						
Isoniazid						
No. of indeterminate results	**Sensitivity**		**Specificity**		**Positive LR (95% CI)**	**Negative LR (95% CI)**
	% (95% CI)	**No. of specimens positive/total no. tested**	**% (95% CI)**	**No. of specimens positive/total no. tested**		
	96.4 (92–98.5)	162/168	96.8 (92.3–98.8)	151/156	30.1 (12.7–71.3)	0.04 (0.02–0.08)
0	97.6 (92.7–99.4)	123/126 (P = 0.69[d])	99.1 (92.7–99.4)	115/116	113.3 (16.1–797.3)	0.03 (0.008–0.07)
9	92.8 (79.4–98.1)	39/42 (P = 0.15,[e] P = 0.08[d])	90 (75.4–96.7)	36/40	9.3 (3.7–23.6)	0.08 (0.03–0.24)

South Africa. The rate of detection of *M. tuberculosis* complex in smear-positive sputum specimens was 97.9%, with seven invalid results. This might be due to the DNA degradation explained by the long-term storage of the DNA of the isolates for several months at −20°C prior to performance of the FluoroType assay. In a routine laboratory, DNA extracts should be analyzed immediately.

The FluoroType MTBDR assay showed a high sensitivity for the detection of mutations conferring resistance to rifampin and isoniazid irrespective of smear gradation. This is an important finding, as the only commercially available WHO-endorsed test for MDR-TB is currently recommended for use on smear-positive specimens only. While no difference in the performance between MTBDR*plus* and FluoroType for the detection of rifampin and isoniazid resistance was observed, a higher indeterminate rate was observed for MTBDR*plus* for the detection of rifampin resistance in smear-negative specimens. Within the group of 322 sputum specimens that tested positive for the *M. tuberculosis* complex with the FluoroType MTBDR assay, 17 FluoroType results were found to be discrepant from the MTBDR*plus* results obtained for the corresponding cultured isolate. When using Sanger sequencing and the MTBDR*plus* result for the sputum specimen, only 4 results discrepant by FluoroType were proven to be truly discordant. For the remaining 13 results discrepant by FluoroType, 6 resistant results matched the MTBDR*plus* result from sputum, implying the loss of the resistant population of the cultured isolate; 2 mutations (*rpoB* Q513P and a TTC insertion in *rpoB* between codons 514 and 515) were undetected by MTBDR*plus* but found by Sanger sequencing; and 5 sensitive results matched the MTBDR*plus* result from sputum, implying a transcription error for the resistant cultured isolates.

Our results showed a sensitivity and a specificity for the detection of rifampin resistance in specimens similar to those obtained by culture of the isolates in combination with phenotypic DST as the reference method (14). However, Hillemann et al. showed a lower sensitivity for the detection of isoniazid resistance (91.7%), which may be explained by different frequencies of noncanonical resistance markers in the respective study populations (14).

Besides the discrimination of rifampin and/or isoniazid resistance, the software also allows the reliable identification of most significant associated mutations in *rpoB*, *katG*, and the *inhA* promoter region. The software was able to detect the correct mutation genotype for *rpoB*, *katG*, and the *inhA* promoter for 97.5%, 96.6%, and 96.6% of the samples, respectively, when using sequencing and MTBDR*plus* as reference methods. Isolates with a few uncommon mutations (e.g., *rpoB* Q513P, *rpoB* TTC insertion between codons 514 to 515, and *inhA* promoter G−17T) were correctly evaluated as mutants but without differentiation of the specific mutation. As specified by the manufacturer, the software-based differentiation of these rare mutations will be possible with the updated versions of the analyzer software. Knowing the exact mutation is crucial, as resistance is not a binary phenomenon and different mutations that are identified by this assay confer a range of resistance phenotypes with large variations in MICs (15–18). Five disputed *rpoB* mutations

TABLE 5 Performance of FluoroType MTBDR for detection of MDR-TB from smear-positive and smear-negative sputum specimens when using Sanger sequencing and GenoType MTBDR*plus* as reference methods[a]

Specimen	Sensitivity			Specificity			Positive LR (95% CI)	Negative LR (95% CI)	No. of indeterminate results
	% (95% CI)	No. of specimens positive/total no. tested		% (95% CI)	No. of specimens positive/total no. tested				
All (n = 315)	99.3 (95.8–100)	148/149		100 (97.2–100)	166/166		Infinity (NaN–infinity)	Infinity (NaN–infinity)	7
Sputum smear positive (n = 228)	99.1 (94.3–100)	108/109		100 (96.1–100)	119/119		Infinity (NaN–infinity)	0.09 (0.001–0.06)	4
Sputum smear negative (n = 87)	100 (89.1–100)	40/40		100 (90.6–100)	47/47		Infinity (NaN–infinity)	Infinity (NaN–infinity)	3

[a]Definitions of abbreviations: ind, indeterminate; CI, confidence interval; LR, likelihood ratio; NaN, the calculation could not be performed because the values entered included one or more instances of zero.

TABLE 6 Accuracy of FluoroType MTDR to detect specific mutations in *rpoB* identified by Genotype MTBDR*plus* and sequencing[a]

	No. of isolates for which the genotype was:							
	Identified by MTBDR*plus* or sequencing	Correctly identified by FluoroType MTBDR	Incorrectly identified by FluoroType MTBDR					% of isolates[b]
rpoB genotype			Indeterminate	False resistance	Incorrect mutation	Undifferentiated mutation	Only WT was recorded	
WT	138	135	2	1				97.8
S531L	135	133			2			98.5
Q513P	1	0				1		0
Het/mixed	11	9				1	1	81.1
Total[c]	322	314						97.5

[a]Definitions of abbreviations: WT, wild type; Het/mixed, heteroresistance and/or mixed infection.
[b]Genotypes identified with 100% accuracy by FluoroType MTBDR: H526D (9/9), H526Y (7/7), D516V (4/4), D516Y (3/3), H526N (3/3), L533P (2/2), insertion between codons 514 and 515 (1/1), L511P (2/2), H526L (2/2), H526R (1/1), S531F (1/1), Q513K (1/1), and H526P (1/1).
[c]The total includes the results for genotypes identified with 100% accuracy.

(L511P, D516V, H526L, H526N, and L533P) that have previously been associated with controversial rifampin phenotypes were correctly identified by FluoroType (17–23). Infections caused by strains harboring the mutations D516V, L533P, and H526L may still be treatable with rifabutin or high-dose rifampin (17, 22, 24, 25). Similarly, infections caused by strains harboring mutations in the promoter region of *inhA* may still be treated with high-dose isoniazid (26, 27). These mutations also provide additional information regarding MDR treatment, as they also confer resistance to ethionamide (28, 29).

The FluoroType assay is a rapid molecular test that can provide operator-independent results within 3 h, which is a time to the result significantly faster than that of the MTBDR*plus* assay, as it does not require any hands-on time after PCR amplification (2 to 3 h for setup and running of the GT-Blotter for MTBDR*plus* hybridization). In addition, the MTBDR*plus* assay requires manual or semiautomated scoring of hybridization patterns, whereas the results of the FluoroType assay are analyzed and reported independently by the analyzer software. The FluoroType assay is also performed in a single closed tube, preventing the release of amplicons and thereby eliminating the risk of laboratory cross contamination and reducing the laboratory infrastructure required to perform molecular-based DST. A limitation of the study was the use of MTBDR*plus* as a reference method. Phenotypic DST would have been the optimal reference method; however, the aim of this study was to evaluate the performance of FluoroType against a WHO-endorsed molecular assay that is routinely used in our setting. Evaluation of future diagnostics studies could use whole-genome sequencing or targeted deep next-generation sequencing to resolve discrepancies, given their superior sensitivity to detect resistant subpopulations.

These findings suggest that the FluoroType MTBDR assay could be implemented as

TABLE 7 Accuracy of FluoroType MTDR to detect specific mutations in *katG* identified by Genotype MTBDR*plus* and sequencing

	No. of isolates for which the genotype was:							
	Identified by Genotype MTBDR*plus* and sequencing	Correctly identified by FluoroType MTBDR	Incorrectly identified by FluoroType MTBDR					% of isolates
katG genotype[a]			Indeterminate	False resistance	Incorrect mutation	Undifferentiated mutation	Only WT was recorded	
WT	257	254	2			1		98.8
S315T1	42	42						100.0
S315T2	14	11			1	2		78.6
S315I	1	0			1			0.0
Het/mixed	8	4				3	1	50.0
Total	322	311						96.6

[a]Definitions of abbreviations: WT, wild type, Het/mixed, heteroresistance and/or mixed infection.

TABLE 8 Accuracy of FluoroType MTDR to detect specific mutations in *inhA* promoter identified by Genotype MTBDR*plus* and sequencing[a]

inhA promoter genotype	No. of isolates in which the genotype was:							
	Identified by Genotype MTBDR*plus* and sequencing	Correctly identified by FluoroType MTBDR	Incorrectly identified by FluoroType MTBDR					% of isolates
			Indeterminate	False resistance	Incorrect mutation	Undifferentiated mutation	Only WT was recorded	
WT	211	207	3	1				98.1
T−15C	100	99	1					99.0
G−17T	4	0			1	3		0.0
T−8A	1	1						100.0
T−8C	1	1						100.0
Het/mixed	5	3	1			1		60.0
Total	322	311						96.6

[a]Definitions of abbreviations: WT, wild type; Het/mixed, heteroresistance and/or mixed infection.

a replacement for the MTBDR*plus* assay, thereby greatly simplifying the rapid diagnosis of rifampin and isoniazid resistance.

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