

Predictive Value of Molecular Drug Resistance Testing of *Mycobacterium tuberculosis* Isolates in Valle del Cauca, Colombia

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Previous evaluations of the molecular GenoType tests have promoted their use to detect resistance to first- and second-line antituberculosis drugs in different geographical regions. However, there are known geographic variations in the mutations associated with drug resistance in *Mycobacterium tuberculosis*, and especially in South America, there is a paucity of information regarding the frequencies and types of mutations associated with resistance to first- and second-line antituberculosis drugs. We therefore evaluated the performance of the GenoType kits in this region by testing 228 *M. tuberculosis* isolates in Colombia, including 134 resistant and 94 pansusceptible strains. Overall, the sensitivity and specificity of the GenoType MTBDR_{plus} test ranged from 92 to 96% and 97 to 100%, respectively; the agreement index was optimal (Cohen's kappa, >0.8). The sensitivity of the GenoType MTBDR_{sl} test ranged from 84 to 100% and the specificity from 88 to 100%. The most common mutations were *katG* S315T1, *rpoB* S531L, *embB* M306V, *gyrA* D94G, and *rrs* A1401G. Our results reflect the utility of the GenoType tests in Colombia; however, as some discordance still exists between the conventional and molecular approaches in resistance testing, we adhere to the recommendation that the GenoType tests serve as early guides for therapy, followed by phenotypic drug susceptibility testing for all cases.

In June 2008, the World Health Organization (WHO) endorsed the use of rapid reverse line blot tests to detect multidrug-resistant tuberculosis (MDR-TB) strains. This step was taken in order to improve the timely detection of MDR-TB, thereby reducing the morbidity and mortality associated with this important public health threat and facilitating efforts to stop the transmission of such strains (1). One of the endorsed rapid molecular tests, the GenoType, consists of two separate reverse line blot assays for detecting mutations associated with drug resistance. The GenoType MTBDR_{plus} test is used to detect mutations associated with resistance to rifampin and isoniazid, and the GenoType MTBDR_{sl} test detects mutations associated with resistance to ethambutol, fluoroquinolones, and second-line injectables.

Previous evaluations of the GenoType kits have documented their performance in detecting resistance to first- and second-line antituberculosis (anti-TB) drugs in different continents, like Africa, Asia, and Europe (2, 3, 4, 5). From these studies, it became clear that there are geographic variations in the mutations that are associated with drug resistance in *Mycobacterium tuberculosis* (6). Especially in South America, there is a paucity of information on the distribution and types of gene mutations associated with drug resistance (7).

Thus, given that the GenoType MTBDR_{plus} and MTBDR_{sl} assays were recently introduced into use in the Latin American regions, our goal was to determine and describe the frequency of mutations associated with drug resistance to first- and second-line anti-TB drugs in Colombia and to complete a performance evaluation of the two tests.

MATERIALS AND METHODS

Study site. This study was conducted at the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) in Cali, Colombia. IRB approval was obtained prior to the initiation of this study.

Clinical strains. Two hundred twenty-eight *M. tuberculosis* strains were selected randomly from a collection of strains that were submitted to or isolated at the CIDEIM to perform drug susceptibility testing between 2001 and 2011. These corresponded to a total of 94 pansusceptible strains

and a group of 134 resistant strains, composed of mono-resistant, MDR, and extensively drug-resistant (XDR) strains, including other resistant combinations. The frozen strains were thawed and cultured on Löwenstein-Jensen slants for genotypic evaluation. The majority of the strains were isolated from patients who were diagnosed in the state of Valle del Cauca, Colombia.

Phenotypic drug susceptibility testing. Phenotypic drug susceptibility testing (DST), which is considered the gold standard in resistance testing, was performed using the indirect agar proportion method (PM) on Middlebrook 7H10 agar (8), with the following antituberculosis drugs and concentrations: 0.2 and 1 µg/ml isoniazid (INH), 1 µg/ml rifampin (RIF), 5 µg/ml ethambutol (EMB), 2 µg/ml ciprofloxacin (CIP) and moxifloxacin (MOX), and 5 µg/ml amikacin (AMK) and kanamycin (KAN). *M. tuberculosis* H37Rv and an XDR clinical strain were used as controls for each group of DST that was processed.

GenoType MTBDR_{plus} and GenoType MTBDR_{sl} testing. The GenoType MTBDR_{plus} test (Hain Lifescience GmbH, Nehren, Germany) detects INH and RIF resistance mutations in the *katG*, *inhA*, and *rpoB* genes. The GenoType MTBDR_{sl} test (Hain Lifescience GmbH, Nehren, Germany) visualizes mutations in the *gyrA*, *rrs*, and *embB* genes that are associated with resistance to fluoroquinolones, second-line injectables, and EMB. The steps for both tests, including the DNA extraction step, were performed according to the manufacturer's instructions. DNA amplification was performed using HotStar_{Taq} DNA polymerase (Qiagen, GmbH, Hilden, Germany) with the recommended number of cycles. Finally, hybridization of the single-stranded biotin-labeled amplicons to membrane-bound probes on the strips, followed by the addition of conjugate and substrate to generate visible hybridization patterns on the strip, were performed using a TwinCubator (Hain Lifescience GmbH, Nehren,

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TABLE 1 GenoType MTBDRplus performance with first-line antituberculosis drugs

GenoType MTBDRplus result	Results of the proportion method	Sensitivity ^a	Specificity ^a	PPV ^{a,b}	NPV ^{a,c}	Cohen's kappa ^a
	Isoniazid (<i>inhA</i> and <i>katG</i>)	94.7 (89.46–97.41)	100 (95.9–100)	100 (97.02–100)	92.78 (85.85–96.46)	0.935 (0.80–1)
	Resistant					
	Susceptible					
Resistant	125					
Susceptible	7					
	Rifampin (<i>rpoB</i>)	95.97 (90.91–98.27)	97.94 (92.79–99.43)	98.35 (94.17–99.55)	95 (88.82–97.85)	0.936 (0.80–1)
	Resistant					
	Susceptible					
Resistant	119					
Susceptible	5					
	MDR status ^d	92.68 (86.68–96.1)	97.96 (92.86–99.44)	98.28 (93.93–99.53)	91.43 (84.51–95.43)	0.899 (0.76–1)
	MDR					
	Non-MDR					
MDR	114					
Non-MDR	9					

^a Data are reported as % (95% CI).

^b PPV, positive predictive value.

^c NPV, negative predictive value.

^d MDR, multidrug-resistant.

Germany). Strips were allowed to dry and were interpreted according to the guide included in the kit. For each gene, the test evaluates the presence of wild-type (WT) and/or mutant (MUT) sequences.

Statistical analysis. Laboratory data were processed and analyzed using SPSS version 20.0 statistical software (SPSS Inc., Chicago, IL). Sensitivity, specificity, negative and positive predictive values, and concordance were calculated applying the Web-based open-source program OpenEpi, using a 95% confidence interval.

RESULTS

Performance of the GenoType MTBDRplus kit. GenoType MTBDRplus correctly identified INH resistance in 125/132 strains, RIF resistance in 119/124 strains, and MDR-TB in 114/123 of the tested strains (Table 1). The test yielded 7 false-negative results in INH-resistant strains; all 7 only revealed hybridization to WT probes and showed no mutations on the resistance probes. No false positives were found in this test. Most mutations (122/125) associated with resistance to INH were observed in the *katG* gene. Only 6% (8/125) of the INH-resistant strains revealed mutations in the *inhA* gene. Moreover, 6 out of those 8 strains also had an additional mutation in the *katG* gene, and thus, only two strains had mutations exclusively in the *inhA* gene. The WT probes for *inhA* were absent for one additional strain without hybridization in the other MUT bands; this strain was also classified as INH resistant.

Regarding the identification of RIF resistance, the test detected five false negatives, and all of them presented a WT pattern. Two false-positive molecular detections of RIF resistance came to light; one of them was classified as resistant because of the absence of a WT7 band yet no MUT bands, and the other strain had a MUT2B (H526D) band. Overall, the test had good performance in the detection of resistance to both INH and RIF and hence the detection of MDR strains, with a sensitivity and specificity of >92% and 96%, respectively. The concordance between the phenotypic and molecular analyses amounted to nearly 0.90.

GenoType MTBDRsl performance. The GenoType MTBDRsl test correctly detected 66/78 strains that were resistant to EMB, 15/17 to CIP, 3/3 to MOX, 27/29 to AMK, and 4/4 to KAN (Table 2). For EMB, the molecular test detected 12 false negatives, all of which exclusively revealed WT bands and an absence of mutations. In addition, two false-positive strains associated with the *embB* gene were detected; these were classified as resistant since they showed the mutations MUT1A-M306I and MUT1B-M306V. Similarly, two false-negative results in the detection of CIP resistance were observed, which were associated with an absence of mutations in the *gyrA* gene, showing only WT bands.

Although eight strains were found to have a false-positive result to MOX, this observation should not be seen as a completely negative feature of the test, as 7/8 of these strains were resistant to CIP. Considering the current critical concentration for MOX, the concordance of the test to detect resistance to MOX should be given more consideration. Nevertheless, it remains important to know whether the causative bacteria of MDR-TB and XDR-TB are still susceptible to at least one fluoroquinolone.

For second-line injectables, only two false-positive results were detected for AMK, and there were no discordances for KAN. Therefore, overall, the GenoType MTBDRsl test had good performance in detecting resistance against EMB, fluoroquinolones, and second-line injectables, with variable, but high, sensitivities and specificities (between 84% and 100% and 88% and 100%, respectively) and variable concordance (between 0.4 and 1).

Frequency of mutations. The most frequent mutations detected by the GenoType MTBDRplus and MTDRsl tests are shown in Table 3. Several less-common mutations were also found: for *rpoB*, H526Y ($n = 11$; 9%), D516V ($n = 9$; 8%), and H526D ($n = 5$; 4%), and for *katG* and *inhA*, S315T2 ($n = 12$; 10%) and T8C ($n = 2$; 2%). Additionally, the D94A (20%; $n = 3$), D94N/Y (13%; $n = 2$), and A90V ($n = 2$; 13%) mutations were observed for *gyrA*,

TABLE 2 GenoType MTBDRsl performance for second-line antituberculosis drugs

GenoType MTBDRsl result	Proportion method results		Sensitivity ^a	Specificity ^a	PPV ^{a,b}	NPV ^{a,c}	Cohen's kappa ^a
	Ethambutol (<i>embB</i>)		84.62 (75.01–90.97)	97.1 (90.03–99.2)	97.06 (89.9–99.19)	84.81 (75.3–91.09)	0.810 (0.65–0.97)
	Resistant	Susceptible					
Resistant	66	2					
Susceptible	12	67					
	Ciprofloxacin (<i>gyrA</i>)		88.24 (65.66–96.71)	100 (96.87–100)	100 (79.61–100)	98.35 (94.17–99.55)	0.929 (0.76–1)
	Resistant	Susceptible					
Resistant	15	0					
Susceptible	2	119					
	Moxifloxacin (<i>gyrA</i>)		100 (43.85–100)	88.57 (79.04–94.09)	27.27 (9.746–56.57)	100 (94.17–100)	0.389 (0.20–0.57)
	Resistant	Susceptible					
Resistant	3	8 ^d					
Susceptible	0	62					
	Amikacin (<i>rrs</i>)		93.1 (78.04–98.09)	100 (96.37–100)	100 (87.54–100)	98.08 (93.26–99.47)	0.955 (0.78–1)
	Resistant	Susceptible					
Resistant	27	0					
Susceptible	2	102					
	Kanamycin (<i>rrs</i>)		100 (51.01–100)	100 (82.41–100)	100 (51.01–100)	100 (82.41–100)	1 (0.58–1)
	Resistant	Susceptible					
Resistant	4	0					
Susceptible	0	18					

^a Data are reported as % (95% CI).

^b PPV, positive predictive value.

^c NPV, negative predictive value.

^d Only one strain is a true false positive; the other seven strains are part of the 15 strains that showed resistance to ciprofloxacin due to common *gyrA* gene-conferred resistance.

as well as the G1484T (4%; $n = 1$) mutation for *rrs* and the M306I ($n = 9$, 14%) mutation for *embB*.

This study revealed the presence of 11 heteroresistant strains (according to GenoType tests): one associated with *katG* heteroresistance, one associated with *inhA*, three associated with *gyrA*, four with *rrs*, one with *embB*, and one case associated with both *inhA* and *rrs* heteroresistance. This heteroresistance is evidenced

TABLE 3 Most frequent mutations associated with *M. tuberculosis* resistance to first- and second-line drugs

Gene mutation probe (mutation)	% of strains (no. of mutated strains/no. of resistant strains detected by GenoType MTBDRplus and MTBDRsl)
<i>rpoB</i> MUT3 (S531L)	64 (76/119) ^a
<i>katG</i> MUT1 (S315T1)	88 (110/125)
<i>inhA</i> MUT1 (C15T)	5 (6/125)
<i>gyrA</i> MUT3C (D94G)	87 (13/15) ^b
<i>rrs</i> MUT1 (A1401G)	96 (27/28) ^c
<i>embB</i> MUT1B (M306V)	83 (55/66)

^a One strain had a double mutation (MUT2A and MUT2B).

^b Four strains had multiple mutations: three strains with double mutations (MUT3B and MUT3C; MUT1 and MUT3C; and MUT3A and MUT3C, respectively) and one strain with three mutations (MUT1, MUT3A, and MUT3C).

^c Twenty-seven strains were resistant to AMK and four to KAN; in this last group, three of the 27 strains were also resistant to AMK, and one strain was resistant only to KAN.

by the simultaneous hybridization of a WT probe and MUT probe on a specific position of a gene.

Susceptibility or resistance in the absence of mutations. Some strains did not show hybridization on any WT or MUT probe (Table 4), a result that according to manufacturer's instructions classifies the strain as resistant. The majority of these cases were RIF resistant, associated with the absence of WT7 in the *rpoB* gene (codons 526 to 529), MUT2A (H526Y), and MUT2B (H526D). Five INH-resistant cases were associated with the absence of WT and MUT probes on the *inhA* gene: four on WT2 (position 8) and one on WT1 (positions 15 and 16). The first case also had the mutation *katG* MUT1 (S315T2) and the second lacked hybridization only on *inhA* WT1 (Table 4). On the contrary, two susceptible cases showed the absence of both WT- and MUT-probe hybridization. One strain, susceptible to RIF, lacked hybridization on the WT7 and MUT2A (H526Y) probes for *rpoB*. Another strain lacked WT3, MUT3A (D94A), MUT3B (D94N/Y), MUT3C (D94G), and MUT3D (D94H) on *gyrA* and was susceptible to MOX, but the susceptibility profile for CIP is unknown.

DISCUSSION

This study is the first, of which we are aware, to describe the mutations associated with *M. tuberculosis* resistance, both to first- and second-line antituberculosis drugs, in strains from Colombia. We

TABLE 4 Absence of hybridization on wild-type and mutation bands

Gene	<i>n</i>	Wild-type probe (codon[s])	Mutation probe (mutation)
<i>rpoB</i> ^a	12	WT7 (526–529) ^b	MUT2A (H526Y)-MUT2B (H526D)
	2	WT8 (530–533)	MUT3 (S531L)
	5	WT2/WT3 (510–517)	MUT1 (D516V)
	2	WT2/WT3 (510–517) and WT3/WT4 (513–519)	MUT1 (D516V)
<i>inhA</i>	1	WT1 (15–16) ^c	MUT1 (C15T)-MUT2 (A16G)
	4	WT2 (8) ^d	MUT3A (T8C)-MUT3B (T8A)
<i>gyrA</i>	1	WT3 (92–97) ^e	MUT3A (D94A)-MUT3B (D94N/Y) MUT3C (D94G)-MUT3D (D94H)
<i>embB</i>	3	WT1 (306) ^f	MUT1A (M306I)-MUT1B (M306V)

^a Strains not associated with additional mutations detected by GenoType in *rpoB*.

^b One of these strains is a false positive. Another strain has double WT probe absence (WT2/WT3 and WT7).

^c Strain with no additional mutations in *inhA* or *katG* as detected by GenoType.

^d Strains with additional mutations in *katG* (MUT1/S315T1).

^e False positive.

^f Strains not associated with additional mutations in *embB* as detected by GenoType.

performed this evaluation using the GenoType MTBDR_{plus} and MTBDR_{sl} tests, which are already in use in multiple countries around the world but were only recently introduced in Colombia. The majority of the strains evaluated were isolated in the Valle del Cauca state, where an MDR-TB hot spot was detected previously (9), in addition to a high proportion of the Beijing genotype among MDR-TB cases (10), and where 10 out of 14 XDR-TB cases were detected (11). Information on the performance of these tests in Colombia is valuable, because the use of rapid molecular tests to detect resistance to antituberculosis drugs has become an important tool in the fight against MDR-TB and XDR-TB. In Colombia, the GeneXpert and GenoType tests are used as parts of different algorithms to detect drug resistance in at-risk populations. Physicians often make therapeutic decisions based first on molecular test results that are available in days and then submit samples or cultures for further analysis or confirmation by phenotypic methods, which usually take weeks. These therapeutic decisions are cost-effective for health systems, as patients start treatment quickly and thus there are fewer possibilities for generating or increasing resistance, which has been correlated with high treatment costs.

In our study, both susceptible and resistant, mainly MDR, *M. tuberculosis* strains were evaluated; unfortunately, only a limited number of strains resistant to second-line drugs were evaluated. We found the performance of the GenoType tests in Colombia to be similar to or even better than those described in studies elsewhere, like in Mexico, Brazil, Peru, Chile, Bolivia, France, Italy, Turkey, Spain, Vietnam, the Russian Federation, Ethiopia, and China (4, 12–23). In general, sensitivity, specificity, positive and negative predictive values, and concordance with phenotypic DST support the utility of these tests for the screening of resistance to first- and second-line antituberculosis drugs in our country. However, as some discordance still exists between the conventional and molecular approaches to DST, we adhere to the recommendation that the GenoType tests, particularly MTBDR_{plus}, should serve as an early guide for therapy, followed by phenotypic DST confirmation for all cases (24).

The discrepancies found in this study might be associated with different factors, such as mutations in alternative genes or changes

in resistance genes that were not detected by the tests. Also, synonymous polymorphisms, or mutations that are not expressed as a resistant phenotypic profile, are recorded as false-positive cases. False negatives were found for INH, RIF, EMB, CIP, and AMK. Alternative resistance genes not included in the tests, such as *ahpC*, *kasA*, and *ndh*, might explain the occurrence of phenotypic resistance to INH in the absence of mutations in the tested genes. Also, mutations not detected in the *gyrB* gene may be related to resistance to CIP. For aminoglycosides and cyclic peptides, some mutations in the *rrs*, *tlyA*, *eis* promoter, and *gidB* genes have been associated with resistance (25), and regarding EMB, other genes, such as *embA*, *embC*, and *embR*, may be important to consider. Moreover, the absence of WT and MUT probes in the hybridization may indicate mutations in positions that are not detected by the MUT probes. In addition, susceptible strains with nonhybridizing WT probes may suggest the presence of synonymous polymorphisms.

The most frequent mutations found in our study were similar to those reported in other studies in Latin America, Europe, Africa, and Asia, which reveals that several regions share a high frequency of the same mutations, but at the same time, these distributions can vary significantly by country. Specifically, the frequency of the *katG* MUT S315T1 in Latin America varied, with 52% in Mexico, 61.9% in Brazil, 71.2% in Peru, and 88% in Colombia (12–14). In comparison, the frequency in some countries on other continents was 65% in France, 66.5% in Italy, 73% in Turkey, 87% in Spain, 88% in Vietnam, 94% in the Russian Federation, and 94% in Ethiopia (17–23).

Likewise, regarding the *rpoB* gene MUT S531L, the percentages of the most frequent mutation detected in several Latin America countries were 47% in Mexico, 56.4% in Peru, 56% in Chile, 59% in Bolivia, and 64% in Colombia (12, 14–16); in other countries not in the Latin American region, the percentage seems to be higher, with 47% in France, 53.4% in Turkey, 58.5% in Italy, 60% in Vietnam, 63% in Spain, 73% in Ethiopia, and 86.9% in the Russian Federation (17–23).

Studies on the distribution of the mutations related to second-line anti-TB drugs are limited in Latin America. Some studies in China, Vietnam, and South Africa revealed that the most frequent mutations are D94G (*gyrA*) (36.5%) and A1401G (*rrs*) (42.1%) in China, D94G (54%) and A1401G (80%) in Vietnam, and D94G (42.4%) in South Africa; these were the most frequent mutations also detected in Colombian strains (4, 21, 26).

This study revealed the presence of heteroresistance, which is defined as the coexistence of susceptible and resistant microorganisms to antituberculosis drugs in the same patient (27). Alternatively, other less-studied molecular mechanisms, such as gene duplications, may play a role. GenoType tests confirmed their advantage over phenotypic DST in detecting this, because DST classifies such strains as susceptible when the patient's sample actually has two subpopulations, susceptible and resistant. This represents a clear advantage of reverse line blot assays. For the time being, however, it remains unclear what this observation implies in terms of treatment adjustment and outcomes.

In conclusion, rapid molecular resistance tests to screen for first- and second-line drug resistance in *M. tuberculosis* strains in Colombia were evaluated favorably, given their high concordance with phenotypic testing and the high predictive value. However, a newer version of the tests with more mutations and/or more genes might be considered to improve their performance. Further anal-

ysis and studies that include sequencing to detect more molecular targets involved in resistance mechanisms will facilitate this process.

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