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First Evaluation of GenoType MTBDR*plus* 2.0 Performed Directly on Respiratory Specimens in Central America

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The turnaround times for conventional methods used to detect *Mycobacterium tuberculosis* in sputum samples and to obtain drug susceptibility information are long in many developing countries, including Panama, leading to delays in appropriate treatment initiation and continued transmission in the community. We evaluated the performance of a molecular line probe assay, the Genotype MTBDR*plus* version 2.0 assay, in detecting *M. tuberculosis* complex directly in respiratory specimens from smear-positive tuberculosis cases from four different regions in Panama, as well as the most frequent mutations in genes conferring resistance to isoniazid (*katG* and *inhA*) and rifampin (*rpoB*). Our results were confirmed with the nitrate reductase assay and genomic sequencing. *M. tuberculosis* complex was detected by the Genotype MTBDR*plus* 2.0 assay with 100% sensitivity and specificity. The sensitivity and specificity for rifampin resistance were 100% and 100%, respectively, and those for isoniazid resistance were 90.7% and 100%. Isoniazid monoresistance was detected in 5.2% of new cases. Genotype MTBDR*plus* 2.0 is highly accurate in detecting *M. tuberculosis* complex in respiratory specimens and is able to discriminate isoniazid-monoresistant cases from multidrug-resistant cases within 2 days.

Novel diagnostic tools and their timely implementation in areas in which disease is endemic are required to make progress toward the goal of reducing the global burden of tuberculosis (TB) (1). In 2008, the World Health Organization (WHO) recommended the use of molecular line probe assays (LPAs) for the rapid detection of multidrug-resistant (MDR) TB (defined as TB resistant to the first-line drugs isoniazid [INH] and rifampin [RIF]). Two years later, the WHO endorsed the use of the Xpert MTB/RIF assay for the rapid detection of MDR TB directly in sputum samples (2); however, its relatively high cost has precluded its widespread use in resource-limited settings. The Genotype MTBDR*plus* 2.0 assay is one of the commercially available LPAs that became available in February 2007 (3). Although this assay has been evaluated and implemented in several countries, its utility has not been formally evaluated in settings in Central America with intermediate TB prevalence.

The primary tool for the diagnosis of pulmonary TB cases in Panama, as in many countries, is staining of sputum samples for acid-fast bacilli (AFB), using the Ziehl-Neelsen method. The National Reference Laboratory (NRL) of Panama employs the Canetti multiple-proportion method for drug susceptibility testing (DST). The sensitivity of sputum AFB staining is approximately 50% and may be as low as 30% in HIV-infected patients (4, 5). Additionally, the turnaround time for DST is at least 6 weeks. The limited sensitivity and long delays for these traditional diagnostic modalities promote the continued transmission of drug-susceptible and drug-resistant TB in the community (6). Only 113 MDR cases were reported officially in Panama between 2001 and 2013 (7), although those figures are likely a gross underestimate of the incidence of MDR TB, since DST is not routinely performed unless needed based on clinical grounds.

The effective use of a molecular technique applied directly to sputum samples, with a rapid turnaround time, is needed to reduce transmission and to avoid outbreaks of MDR TB among vulnerable populations (8). In this study, we evaluated and compared the sensitivity and specificity of the Genotype MTBDR*plus* 2.0 assay, performed directly with AFB-positive respiratory specimens, with conventional culture-based diagnostic methods and DST. Genomic sequencing of all isolates was performed to corroborate the LPA results.

MATERIALS AND METHODS

Ethics statement. This study was approved by the Bioethics Committee of Panama.

Respiratory samples. Sixty-eight smear-positive specimens were collected by the below-named individuals at six different hospitals from three health regions in Panama with a high prevalence of LAM9-c1 MDR isolates in the past decade (6), i.e., Manuel Amador Guerrero Hospital (Colón region) (Mariela Vergara); Nuevo Veranillo Health Center (San Miguelito region) (Silvana Campos); and Complejo Hospitalario Metropolitano Dr. Arnulfo Arias Madrid (Berta Marshall), 24 de Diciembre Hospital (Julio Dominguez), Pueblo Nuevo Health Center (Delsa Pimentel), and Santo Tomás Hospital (Erika Santiago) (all in the Panama City metropolitan region). Most respiratory specimens were from pretreatment cases and were collected between December 2012 and December 2013. Due to financial constraints, smear-negative samples were not evaluated.

Sample processing and culture. Direct smears were prepared from the specimens using Ziehl-Neelsen staining. Smears were read and interpreted in accordance with Pan American Health Organization (PAHO)

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TABLE 1 Demographic data ($n = 68$)

Characteristic	Finding
Age (mean [range]) (yr)	42 (16–90)
Sex (no. [%])	
Female	23 (33.8)
Male	45 (66.1)
TB history (no. [%])	
New case	58 (85.3)
Retreatment	10 (14.7)
HIV infected (no. [%])	
Yes	4 (5.8)
No	33 (48.5)
Unknown	31 (45.5)
Health region (no. [%])	
Colón	40 (58.8)
Panama City metropolitan	23 (33.8)
San Miguelito	5 (7.4)

guidelines (9). Specimens were decontaminated with the standard *N*-acetyl-L-cysteine (NALC)-NaOH method (9), and the pellets were suspended in 1.0 ml of phosphate buffer (pH 6.8). This was followed by inoculation on Lowenstein-Jensen agar, and the residual portions of decontaminated samples were used to perform genomic extraction using a Genolyse kit.

Biochemical characterization and drug susceptibility testing. After incubation on Lowenstein-Jensen agar, isolates were classified as *Mycobacterium tuberculosis* using biochemical assays, including assays of nitrate reduction, niacin production, and catalase inhibition at 68°C. DST was performed with all isolates using the nitrate reductase assay (NRA) and the following critical concentrations of antibiotics: 0.2 μg/ml isoniazid and 40 μg/ml rifampin (10, 11). In cases with discordant results from the LPA and NRA assays, the Canetti multiple-proportion method was performed according to Panama National TB Control Program guidelines (10), using the same antibiotic concentrations as for the NRA assay.

GenoType MTBDR*plus* 2.0 assay. The Genotype MTBDR*plus* version 2.0 test (Hain Lifescience, Germany) is based on DNA strip technology, with three steps, i.e., DNA extraction, multiplex amplification with biotinylated primers, and reverse hybridization, according to the manufacturer's instructions (3). DNA extraction was performed with a Genolyse kit, in three steps, i.e., centrifugation, lysis at 95°C, and neutralization. The following amplification protocol was used for clinical specimens: 1 cycle of 15 min at 95°C, 20 cycles of 30 s at 95°C and 2 min at 65°C, 30 cycles of 25 s at 95°C, 40 s at 50°C, and 40 s at 70°C, and finally 1 cycle of 8 min at 70°C. Reverse hybridization was performed with an automated hybridization system; an Auto-Lipa 48 system (Innogenetics) was used with the reagents provided in the Genotype MTBDR*plus* 2.0 kit for the hybridization procedure (3). Interpretation was performed according to the guide included with the Genotype MTBDR*plus* kit. The absence of a wild-type band and the presence of a mutant band for a specific gene on the strip implied resistance.

Genomic sequencing. Mycobacterial isolates were sequenced with an Illumina HiSeq 2500 instrument, using a paired-end sequencing strategy. DNA samples were extracted from colonies using a QIAamp DNA minikit, sheared into ~250-bp fragments using a Covaris sonicator (Covaris, Inc.), and prepared using a standard whole-genome DNA sequencing sample preparation kit (Illumina, Inc.). Paired-end reads of 72 bp were collected. Base calling was performed using OLB 1.9.3 (Illumina, Inc.). Genome assembly was performed using custom software that implements a comparative assembly approach (12) by aligning reads with the genome sequence of *M. tuberculosis* H37Rv (GenBank accession no. NC_000962.2). The mean depth of coverage over the genome averaged 68-fold over all samples (range, 9.8-fold to 125.6-fold). A local contig-building algorithm was used to identify indels (insertions or deletions) in regions where coverage was low.

RESULTS

Demographic data. During the period of December 2012 to December 2013, 68 smear-positive respiratory samples were collected as part of routine TB diagnostic evaluations (Table 1). The majority of samples were from the region of Colón (40/68 samples [58.8%]) or the Panama City metropolitan area (23/68 samples [33.8%]); 5/68 samples (7.4%) were from the San Miguelito region. The majority of samples (45/68 samples [66%]) were from male subjects. The age range of the subjects was 16 to 90 years, although there were two peaks in the age distribution (18 to 27 years and 48 to 57 years), and the mean age was 42 ± 17.13 years. Ten patients (14.7%) had a history of previous TB treatment, while 58 cases (85.3%) were new TB cases. Four subjects (5.8%) were HIV infected, 33 subjects (48.5%) were not HIV infected, and the HIV status was not known for 31 subjects (45.5%).

Respiratory specimen characteristics. Refrigeration times for the samples varied from 1 to 40 days, due to the distance of some institutions from the NRL. Fewer than one-half of the specimens for which information was available (28/65 specimens [43%]) were refrigerated for 10 days or less prior to processing. Samples were classified as mucopurulent, bloody, mucous, or salivary according to the PAHO classification (9); the majority of samples (37/68 samples [55%]) were determined to be mucous. The volumes of expectorated specimens ranged from 0.5 ml to 4 ml, and three specimens derived from bronchial aspirates ranged from 5 to 10 ml in volume. The most frequently collected volume was 1 ml (26/68 samples [38.2%]). All samples were subjected to acid-fast smear examination, which revealed a range of bacillary loads, as follows: scanty, 7/68 samples (10.3%); 1+, 35/68 samples (51.5%); 2+, 13/68 samples (19.1%); 3+, 13/68 samples (19.1%) (Fig. 1).

Characterization of *M. tuberculosis* complex. Of the 68 AFB smear-positive respiratory specimens, 54 cultures that were positive on Lowenstein-Jensen agar were obtained and classified as *M. tuberculosis* using biochemical assays, including assays of nitrate reduction, niacin production, and catalase inhibition at 68°C (10). The results were confirmed genetically

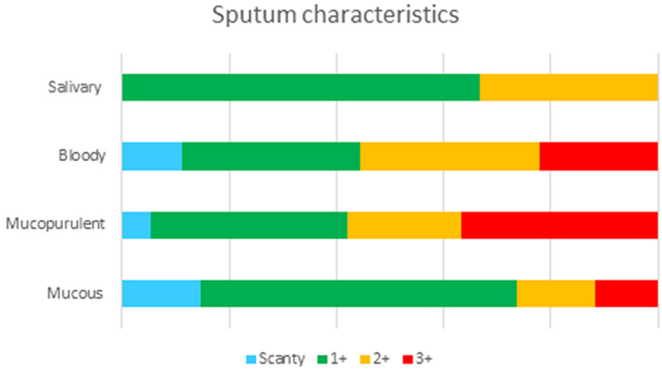

FIG 1 Respiratory specimen characteristics. Samples were classified according to the PAHO classification (9). Colors refer to the proportions of specimens in each quality category, which were graded as scanty, 1+, 2+, or 3+ by acid-fast staining.

TABLE 2 Band patterns of drug-resistant *Mycobacterium tuberculosis* isolates using Genotype MTBDR*plus* 2.0

Gene and band[a]	Gene region or mutation	No. of isolates (%)		
		MDR ($n = 5$)	INH monoresistant ($n = 4$)	RIF monoresistant ($n = 4$)
rpoB				
WT7	526–529	0 (0)	0 (0)	3 (75)
WT8	530–533	5 (100)	0 (0)	1 (25)
MUT2B	H526D	0 (0)	0 (0)	3 (75)
MUT3	S531L	5 (100)	0 (0)	1 (25)
katG				
WT	315	4 (80)	0 (0)	0 (0)
MUT1	S315T1	4 (80)	0 (0)	0 (0)
inhA				
WT1	−15/−16	1 (20)	4 (100)	0 (0)
MUT1	C−15T	1 (20)	4 (100)	0 (0)

[a] WT, wild-type; MUT, mutant.

using DNA sequencing. In contrast, the Genotype MTBDR*plus* 2.0 assay identified *M. tuberculosis* complex in all 68 processed respiratory samples, including the 14 samples for which mycobacteria did not grow on solid agar.

Drug susceptibility testing with nitrate reductase assay. The nitrate reductase assay is used to detect antibiotic resistance following the addition of 1 mg/ml potassium nitrate to Lowenstein-Jensen medium, as the reduction of nitrate can be detected by the development of a reddish color after the addition of the Griess reagent (11). The WHO recommends that the NRA be used directly on smear-positive sputum specimens or on *M. tuberculosis* isolates grown on solid agar (13). Since nitrate reduction, rather than grossly visible colonies, is used as an indicator of growth, the NRA reduces the turnaround time for DST, compared to conventional methods.

In the current study, all positive cultures were tested with the NRA, which yielded the following results: 4 cultures were isoniazid monoresistant, 1 was rifampin monoresistant, 6 were MDR, and 42 were susceptible to both drugs. However, bacilli were not cultivable for 14 samples. Four of those smear-positive samples were obtained following the initiation of treatment, and the remaining 10 isolates were refrigerated for a mean duration of 15.7 days, which perhaps contributed to reduced bacillary viability. Among the 14 samples that did not have reportable phenotypic DST results, the Genotype MTBDR*plus* 2.0 assay detected 2 with rifampin monoresistance, while 12 were susceptible to both drugs.

Drug susceptibility testing with GenoType MTBDR*plus* 2.0. Genotype MTBDR*plus* 2.0 identified 5/68 isolates (7.4%) as MDR, 4/68 (5.8%) as isoniazid monoresistant, and 4/68 (5.8%) as rifampin monoresistant. The C−15T *inhA* mutation was the only mutation detected in all 4 isoniazid-monoresistant isolates. Rifampin-monoresistant samples displayed two types of mutations, i.e., the *rpoB* gene mutations H526D (3/4 isolates) and S531L (1/4 isolates). In contrast, mutations detected in MDR isolates were *rpoB* S531L/*katG* S315T (4/5 isolates) and *rpoB* S531L/*inhA* C−15T (1/5 isolates) (Table 2). Among the 42 culture-positive samples found to be drug susceptible by the NRA, Genotype MTBDR*plus* 2.0 did not detect any mutation in the hotspots of the genes *rpoB*, *katG*, or *inhA*.

Relative to conventional DST, the Genotype MTBDR*plus* 2.0 assay yielded a sensitivity, specificity, positive predictive value, and negative predictive value of 100%, 100%, 100%, and 100%, respectively, for rifampin resistance. The sensitivity, specificity, positive predictive value, and negative predictive value of the Genotype MTBDR*plus* 2.0, relative to DST, were 90.9%, 100%, 100%, and 50%, respectively, for isoniazid resistance.

Genomic sequencing. For three samples, the extracted DNA concentration was below the threshold for performing sequencing. Genomic sequencing was performed for all culture-positive samples and revealed 3 INH-monoresistant strains, 1 RIF-monoresistant strain, and 4 MDR strains. The sensitivity and specificity of the Genotype MTBDR*plus* 2.0 assay, compared to genomic sequencing, were 87.5% and 100% for isoniazid and 100% and 100% for rifampin, respectively. Table 3 shows the correlation between Genotype MTBDR*plus* 2.0, genomic sequencing, and conventional DST results.

DISCUSSION

To our knowledge, our study is the first to evaluate the performance of the Genotype MTBDR*plus* 2.0 assay applied directly to respiratory specimens in Central America, a region of intermediate TB endemicity. In Panama, AFB staining and culture-based DST remain the primary tools for the diagnosis of pulmonary TB and detection of drug resistance. Therefore, parameters such as the quality of the specimen, volume, and storage time are taken into consideration in interpreting microscopy and DST results (10). Importantly, in the current study, the Genotype MTBDR*plus* 2.0 assay was able to detect TB and drug resistance correctly regardless of sample volume or refrigeration time, likely because of the increased sensitivity of DNA amplification-based methods and the lack of requirement for the presence of viable and/or cultivable bacilli in respiratory specimens.

Of the 10 patients with a history of TB treatment, 9 harbored isolates with resistance to ≥1 drug, while 1 isolate was sensitive to both isoniazid and rifampin. Five (56%) of those patients had a history of substance abuse (including cocaine, marijuana, and/or alcohol abuse), and 2 patients were HIV infected. In Panama, as in many parts of the world, drug-resistant TB is prevalent among homeless individuals and those with a history of illicit drug use,

TABLE 3 Summary of results of RIF and INH resistance testing with Genotype MTBDR*plus* assay, sequencing, and conventional DST

Sample	Result[a]					
	MTBDR*plus*		Sequencing		NRA DST	
	RIF^R	INH^R	RIF^R	INH^R	RIF^R	INH^R
003-061	S531L		S531L	Y337C	R	R
006-02	S531L	C−15T	S531L	C−15T	R	R
011-02	H526D		CONT	CONT	CONT	CONT
044-04		C−15T		C−15T	S	R^W
049-01		C−15T	LC	LC	S	R
058-01	S531L	S315T	LC	LC	R	R
098-03	H526D		H526D		R	S
113-061	S531L	S315T	S531L	S315T	R	R
121-01	S531L	S315T	S531L	S315T	R	R
147-04		C−15T		C−15T	S	R
156-061	S531L	S315T	LC	LC	R	R
241-01	H526D		NG	NG	NG	NG
242-062		C−15T		C−15T	S	R

[a] R, resistant; CONT, contamination; LC, low concentration of DNA; NG, no growth; W, weak reaction.

because such populations are less likely to adhere to medical treatment and are at increased risk for acquiring primary drug-resistant TB infections (14, 15).

Among newly diagnosed, culture-confirmed, TB cases, 1 isolate (1.7%) was MDR and 5.2% (3/58 isolates) were found to have isoniazid monoresistance. The prevalence of isoniazid-resistant TB among newly diagnosed cases ranges geographically from 5 to 25% (16). Although the treatment outcomes for such cases remain unclear, several retrospective studies suggest that further drug resistance is promoted in the community when isoniazid resistance is not recognized and standard treatment is provided (17–20). Interestingly, the C−15T mutation, which is significantly less common than the *katG* S315T mutation in isoniazid-resistant isolates worldwide (21) and in Panama, was present in all 3 cases in our study, suggesting the possibility of recent transmission.

The sensitivity of the Genotype MTBDR*plus* 2.0 assay for isoniazid resistance (90.7%) in the current study was greater than that in studies performed prior to 2007 (22), likely because the newer version of the assay analyzes the *inhA* locus in addition to *katG*. The sensitivity of Genotype MTBDR*plus* 2.0 for isoniazid resistance might be further improved by including additional resistance mutations based on geographic distribution (23). Knowledge of the precise mutation conferring resistance may be very useful clinically, particularly in the case of isoniazid, as the *inhA* C−15T mutation generally confers low-level resistance, which may be overcome with higher doses of isoniazid (24, 25). Moreover, this version has been evaluated with smear-negative/culture-positive specimens, showing sensitivities of 58% to 76% (26, 27).

The LPA and DST yielded concordant results for all 42 susceptible isolates. On the other hand, LPA and DST yielded discordant results for two resistant cases. Sample 003-061 was reported as sensitive to isoniazid by the Genotype MTBDR*plus* 2.0 assay and resistant by the NRA, which was confirmed by the Canetti multiple-proportion method. Sequencing of this sample revealed the mutation Y337C in the *katG* gene. Enzymology studies show that this mutation confers resistance by reducing the efficiency of INH radical formation while maintaining catalytic efficiency for the native catalase activity, similar to S315T (28). Although it is not a commonly recognized mutation (29), Y337C has been reported to confer INH resistance among non-clustered isolates (30). Our previous studies revealed that approximately 16% of clustered MDR isolates identified in Panama over the past decade lacked mutations in the *katG* and *inhA* loci (31). The second case with discordant results involved isolate 044-04, which contained a C−15T mutation detected with the LPA, although an indeterminate reaction was noted with the NRA. The Canetti multiple-proportion method yielded the growth of a single isoniazid-resistant colony. Sequencing of this sample confirmed the LPA result. Interestingly, this sample had been refrigerated for 18 days, which suggests that there might have been loss of bacterial viability, potentially accounting for the false-negative DST result.

The ability to detect very few resistant bacilli in a sputum sample may represent an advantage of molecular drug susceptibility testing. Although the current version of the Genotype MTBDR*plus* 2.0 assay still cannot detect all INH resistance-conferring mutations, it has a high sensitivity for detecting RIF resistance and thus for identifying MDR cases. Furthermore, the short turnaround time of 2 days for producing TB drug susceptibility results, compared to 2 to 3 months for standard, culture-based, DST methods, is a major boon to facilitate clinical decision-making and the selection of appropriate antitubercular therapy in real time.

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