First evaluation of GenoType MTBDRPlus 2.0 performed directly on respiratory specimens in Central America

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ABSTRACT

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

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INTRODUCTION

Novel diagnostic tools and their timely implementation in endemic areas are required to make progress towards 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 and rifampicin. Two years later, the WHO endorsed the use of Xpert MTB/RIF 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 MTBDRplus 2.0 assay is one of the commercially available LPAs which 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 intermediate TB prevalence settings in Central America.

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; Ziehl Neelsen method). The National Reference Laboratory of Panama (NRL) 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 a minimum of 6 weeks. The limited sensitivity and long delay in 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 2001-2013 (7), although these figures are likely a gross underestimate of the incidence of MDR TB since DST is not routinely performed unless suspected based on clinical grounds.

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

RESULTS

Demographic data

During the period December 2012 to December 2013, 68 respiratory smear-positive samples were collected as part of routine TB diagnostic workup (Table 1). The majority of samples were from the region of Colón (40/68; 58.8%) or the Panama city metropolitan area (23/68; 33.8%), and 5/68 (7.4%) were from San Miguelito. The majority of samples (45/68; 66%) were from male subjects. The age range of subjects was 16 to 90 years, although there were two peaks in the age distribution (18-27 and 48-57 years old), and the mean age was 42 $\pm$ 17.13. Ten patients (14.7%) had a history of previous TB treatment, while 58 (85.3%) were new TB cases. Four subjects (5.8%) were HIV-infected, and 33 (48.5%) were HIV-uninfected, although the HIV status was unknown in 31 subjects (45.5%).

Respiratory specimen characteristics

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Refrigeration time of the samples varied from 1 to 40 days due to distance of some institutions to the NRL. A little less than half of the specimens (27.9%) had been refrigerated between 6 to 10 days before being processed. The remainder was processed immediately. Samples were classified as mucopurulent, bloody, mucous and salivary according to the Pan American Health Organization (PAHO) classification (9); the majority (37/68; 55%) was determined to be mucous. The volume of expectorated specimens ranged from 0.5ml to 4ml, although 3 specimens derived from bronchial aspirates ranged from 5 to 10ml in volume. The most frequently collected volume (26/68; 38.2%) was 1 ml. All samples were subjected to acid-fast smear examination, revealing a range of bacillary loads: scanty (7/68; 10.3%), 1+ (35/68; 51.5%), 2+ (13/68; 19.1%) and 3+ (13/68; 19.1%) (Figure 1).

Characterization of the *M. tuberculosis complex*

Of the 68 AFB smear-positive respiratory specimens, 54 positive cultures on Lowenstein Jensen agar were obtained and classified as *M. tuberculosis* using biochemical assays, including nitrate reduction, niacin production and catalase inhibition at 68ºC (10). These results were confirmed genetically using DNA sequencing. In contrast, the Genotype MTBDRplus 2.0 assay identified *M. tuberculosis* complex in all 68 processed respiratory samples, including the 14 samples in which mycobacteria did not grow on solid agar.

Drug susceptibility testing with nitrate reductase assay (NRA)

The nitrate reductase assay is used to detect antibiotic resistance following addition of potassium nitrate (KNO3) 1 mg/ml in Lowenstein–Jensen medium, as the

reduction of nitrate can be detected by the development of a reddish color after 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 (12). Since nitrate reduction is used as an indicator of growth rather than grossly visible colonies, the NRA reduces the turnaround time for DST compared to conventional methods.

In the current study, all positive cultures were tested by NRA, yielding the following results: 4 were isoniazid mono-resistant, 1 was rifampicin mono-resistant, 6 were MDR and 42 were susceptible to both drugs. However, bacilli were not cultivable in 14 samples. Four of these smear-positive samples were obtained following initiation of treatment, and the remaining 10 isolates were refrigerated for a mean duration of 15.7 days, perhaps contributing to reduced bacillary viability. Of the 14 samples that did not have a reportable phenotypic DST result, Genotype MTBDRplus 2.0 detected 2 with rifampicin mono-resistance, while 12 were susceptible to both drugs.

Drug susceptibility testing with GenoType MTBDRplus ver 2.0

Genotype MTBDRplus 2.0 identified 5/68 (7.4%) isolates as MDR, 4/68 (5.8%) as isoniazid mono-resistant, and 4/68 (5.8%) as rifampicin mono-resistant. The C-15T *inhA* mutation was the only mutation detected in all 4 isoniazid mono-resistant isolates. Rifampicin mono-resistant samples displayed two types of mutations: *rpoB* gene mutations H526D (3/4) and S531L (1/4). In contrast, mutations detected in MDR isolates were: *rpoB* S531L/*katG* S315T (4/5), and *rpoB* S531L/*inhA* C-15T (1/5) (Table 2).

Among the 42 culture-positive samples found to be drug-susceptible by NRA, Genotype MTBDRplus 2.0 did not detect any mutation in the hotspots of the genes *rpoB, katG* or *inhA*.

Relative to conventional DST, the Genotype MTBDRplus 2.0 yielded a sensitivity, specificity, positive predictive value, and negative predictive value of 100%, 100%, 100%, and 100% for rifampicin resistance. Conversely, the sensitivity, specificity, positive predictive value, and negative predictive value of the Genotype MTBDRplus 2.0 relative to DST were 90.9%, 100%, 100%, 50% for isoniazid resistance.

Genomic sequencing

In five samples the extracted DNA concentration was below the threshold to perform sequencing. Genomic sequencing was performed for all culture-positive samples revealing: 3 INH-monoresistant, 1 RIF-monoresistant and 4 MDR strains. The sensitivity and specificity of Genotype MTBDRplus version 2.0 compared to genomic sequencing for isoniazid was 87.5% and 100%, respectively, and 100% and 100%, respectively, for rifampicin. Table 3 shows the correlation between Genotype MTBDRplus 2.0, genomic sequencing and conventional DST.

Discussion

To our knowledge, our study is the first to evaluate the performance of the Genotype MTBDRplus 2.0 assay applied directly on 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 quality of the specimen, as well as volume and storage time, are taken into consideration for interpreting microscopy and DST results (10). Importantly, in the current study, the Genotype MTBDRplus 2.0 assay was able to correctly detect TB cases and drug resistance regardless of sample volume or refrigeration storage time, likely due to increased sensitivity of DNA amplification-based methods, as well as lack of requirement for the presence of viable and/or cultivable bacilli in respiratory specimens.

Of the 10 patients with a prior history of TB treatment, 9 harbored isolates with resistance to at least one drug, while 1 was sensitive to both isoniazid and rifampicin. Five (56%) of these patients had a history of substance abuse, including cocaine, marijuana, and/or alcohol, and 2 patients were HIV-infected. As in many parts of the world, in Panama drug-resistant TB is prevalent among the homeless and those with a history of illicit drug use, as these populations are less likely to adhere to medical treatment and are at increased for acquiring primary drug-resistant TB infection (13, 14).

Among newly diagnosed, culture-confirmed TB cases, one isolate (1.7%) was MDR and 5.2% (3/58) were found to have isoniazid mono-resistance. The prevalence of isoniazid-resistant TB among newly diagnosed cases ranges geographically from 5-25% (15). Although the treatment outcome for such cases remains 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 (16-19). Interestingly, the c-15t mutation, which is significantly less common than the *katG* S315T mutation in isoniazid-resistant isolates worldwide (20) and in Panama, was present in all 3 cases in our study, suggesting the possibility of recent transmission.

In the current study, the sensitivity of the Genotype MTBDRplus version 2.0 for isoniazid resistance (90.7%) was greater than in studies done prior to 2007 (21) likely because the newer version of the assay also analyzes the *inhA* locus in addition to *katG*. The sensitivity of Genotype MTBDRplus 2.0 for isoniazid resistance might be further improved by including additional resistance mutations based on geographic distribution (22). 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 (23, 24). Moreover, this version has been evaluated in smear-negative/culture positive specimens showing sensitivities between 58% to 76% (25, 26).

The LPA and DST yielded concordant results in all 42 susceptible isolates. On the other hand, LPA and DST yielded discordant results in two resistant cases. Sample 003-061 was reported as sensitive to isoniazid by Genotype MTBDRplus 2.0 and resistant by NRA, which was confirmed by Canetti's 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 efficiency of INH radical formation while maintaining catalytic efficiency for its native catalase activity, similar to S315T (27). Although it is not a commonly recognized mutation (28), Y337C has been reported to confer INH resistance among non-clustered isolates (29). Our previous studies revealed that approximately 16% of clustered MDR isolates in Panama over the past decade lacked mutations at the *katG* and *inhA* loci (30). The second discordant case was isolate 044-04, which contained a c-15t mutation detected by LPA, although an indeterminate reaction was noted by NRA. Canetti's multiple proportion method yielded the growth of a single isoniazid-resistant colony. Sequencing of this sample confirmed the LPA result. Interestingly, this sample was refrigerated for 18 days,

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suggesting there may 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 MTBDRplus 2.0 assay still cannot detect all INH resistance-conferring mutations, it has a high sensitivity for detecting RIF resistance, and, therefore, for identifying MDR cases. Furthermore, the rapid turnaround time of 2 days in yielding TB drug susceptibility results as compared to 2-3 months for standard, culture-based DST methods is a major boon for, facilitating clinical decision-making and the selection of appropriate anti-tubercular therapy in real time.

Materials and methods

Ethics statement

This study was approved by the Bioethics Committee of Panama.

Respiratory samples

68 smear-positive respiratory specimens were collected at four different hospitals from three health regions in Panamá with a high prevalence of the LAM9-c1 MDR isolates in the past decade (6): Manuel Amador Guerrero Hospital (Mariela Vergara; Colón Region); Nuevo Veranillo Health Center (Silvana Campos; San Miguelito Region); 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 Santigo) (all in Panamá

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Metro Region). Most respiratory specimens were from pre-treatment cases collected between December 2012 to 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 PAHO guidelines (9). Specimens were decontaminated with the standard NALC-NaOH method (9) and the pellet was suspended in 1.0ml of phosphate buffer (pH 6.8). This was followed by inoculation on Lowenstein Jensen agar and the residual portion of decontaminated sample was used to perform genomic extraction using the Genolyse kit.

Biochemical characterization and drug susceptibility testing

After incubation on Lowenstein Jensen agar, isolates were classified as *M. tuberculosis* using biochemical assays, including nitrate reduction, niacin production, and catalase inhibition at 68ºC. DST was performed on all isolates using NRA and the following critical concentrations of antibiotics: 0.2 µg/ml isoniazid and 40 µg/ml rifampicin (10, 11).

In cases yielding discordant results between the LPA and NRA assays, Canetti's multiple proportions method was performed according to the Panama National TB Control Program guidelines (10), using the same antibiotic concentrations as the NRA assay.

GenoType MTBDRplus 2.0

Genotype MTBDRplus version 2.0 test (Hain Lifescience, Germany) is based on the DNA STRIP technology consisting of three steps: DNA extraction, multiplex amplification with biotinylated primers and reverse hybridization as per manufacturer's instructions (3). DNA extraction was performed with Genolyse kit in three steps: centrifugation, lysis at 95ºC and neutralization. The following amplification protocol was used for clinical specimens: one cycle of 15 min at 95ºC, followed by 20 cycles of 30 sec at 95ºC and 2 min at 65ºC, followed by 30 cycles of 25 sec at 95ºC, 40 sec at 50ºC and 40 sec at 70ºC, ending with 1 cycle of 8 min at 70ºC. Reverse hybridization was performed with an automated hybridization machine: Auto-Lipa 48 (Innogenetics) was used with the reagents provided according to Genotype MTBDRplus ver 2.0 kit for the hybridization procedure (3). Interpretation was done according to the guide included in the Genotype MTBDRplus kit. The absence of a wild type (WT) band and the presence of a mutant band (MUT) for a specific gene on the strip implied resistance.

Genome Sequencing

Mycobacterial isolates were sequenced on an Illumina HiSeq 2500 instrument using a paired-end (PE) sequencing strategy. DNA samples were extracted from colonies using the QIAamp DNA Mini Kit, sheared into ~250 bp fragments using a Covaris sonicator (Covaris, Inc.), and prepared using the standard whole-genome DNA sequencing sample preparation kit (Illumina, Inc.). Paired-end reads of length 72 bp were collected. Base-calling was performed using Online Base-Caller (OLB) v. 1.9.3 (Illumina, Inc.). Genome assembly was performed using custom software that implements a comparative assembly approach (31) by aligning reads to the genome

sequence of *M. tuberculosis* H37Rv (NC_000962.2). Mean depth of coverage over the genome averaged 68-fold over all samples (range 9.8-125.6). A local contig-building algorithm was used to identify indels (insertions or deletions) in regions where coverage spiked low.

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Author Contributions

Conceived and designed the experiments: FL, TRI, PCK; Performed the experiments: FL, TRI, HS, WA; Analyzed the data: FL, TRI, HS, WA, PCK. Contributed reagents/analysis tools: FL, TRI, PCK. Prepared the manuscript: FL, TRI, PCK.

Table 1: Demographic Data

Demographic data	n=68 (%)
Age	
Mean years (range)	42 (16-90)
Sex	
Female	23 (33.8)
Male	45 (66.1)
TB history	
New cases	58 (85.3)
Retreatment	10 (14.7)
HIV-infected	
Yes	4 (5.8)
No	33 (48.5)
Unknown	31 (45.5)
Health region	
Colón	40 (58.8)
Panamá Metro	23 (33.8)
San Miguelito	5 (7.4)

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315 **Table 2. Band patterns of drug-resistant *Mycobacterium tuberculosis* isolates using**

316 **Genotype MTBDRplus 2.0**

Gene	Band	Gene region or mutation	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	C15T	1 (20)	4 (100)	0 (0)

317

318 **Table 3. Summary of results of RIF and INH resistance by Genotype MTBDRplus,**

319 **Sequencing and conventional DST**

ID SAMPLE	GENOTYPE MTBDRPLUS		GENOMIC 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

320 R: resistant; CONT: contamination; LC: low concentration of DNA; NG: No growth;

321 W: weak reaction.

322 **Figure legends**

323 **Figure 1.** Respiratory specimen characteristics**.** Samples were classified according to
324 the Pan American Health Organization (PAHO) classification (10). Colors refer to the
325 proportion of specimens in each quality category, which are graded as scanty, 1+, 2+, or
326 3+ by acid-fast staining.

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