Direct Molecular Characterization of Acid-Fast Bacilli Smear of Nontuberculosis *Mycobacterium* Species Causing Pulmonary Tuberculosis in Guna Yala Region, Panama

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Abstract. *Mycobacterium tuberculosis* (MTB) stands out as the main causative agent of pulmonary tuberculosis (TB). However, nontuberculous mycobacteria (NTM) species also have the potential to infect and cause TB in susceptible individuals. The objective of this study was to identify NTM species that cause public health problems in remote areas. The study was carried out using 105 sputum smears obtained from patients from the Guna Yala Region of Panama with clinical signs suggestive of TB. DNA was extracted from sputum smears. Nontuberculous mycobacteria and MTB were characterized using polymerase chain reaction restriction analysis (*hsp65*, *rpob*) and an evaluation of 24-mycobacterial interspersed repetitive units–variable number of tandem repeats loci. Twenty-six *Mycobacterium* species were characterized; 19 (18%) were identified as MTB, and 7 (6.7%) were identified as NTM (four *M. avium* complex, two *M. haemophilum*, one *M. tusciae*). These results suggest that at least one in five cases of pulmonary TB among this population is caused by an NTM. Thus, identifying the bacteria causing pulmonary disease is key even in remote regions of the world where standard diagnosis and culture are not available. Strengthening the laboratory capacity within the Guna Yala Region is needed to identify NTM infections promptly.

Tuberculosis (TB) remains a significant public health challenge; 23% of the world population is estimated to have a latent TB infection; these individuals are at risk of developing active TB disease during their lifetime.^{1,2} Although *Mycobacterium tuberculosis* is the most important causative agent of TB, nontuberculous mycobacteria (NTM) may play an important role in the etiology of TB-like syndromes. The number of mycobacterial species recognized as potential pathogens is increasing, and in the past year, an increase in NTM isolation rates has been observed worldwide.^{3,4} Nontuberculous mycobacteria are opportunistic pathogens that are acquired from environmental reservoirs (water, soil) and often represent an important cause of morbidity and mortality in immunocompromised humans.^{3,5}

In Central America, Panama stands as the country with the highest TB rate (52 cases per 100,000 population),¹ and the Guna Yala Region (GYR) is the area with the highest incidence (191.8 per 100,000 population).⁶ The GYR is located in northeastern Panama and is characterized by its lengthy Caribbean coastline inhabited by indigenous people (Figure 1). Determining the incidence and prevalence of NTM lung disease in this area remains problematic primarily because disease reporting is not mandatory and because individual NTM isolates must be assessed to determine their clinical significance. This is in contrast to *M. tuberculosis*, for which each isolate is assumed to be associated with true disease. Therefore, the prevalence of NTM isolates is not known with certainty.

Molecular epidemiology based on polymerase chain reaction (PCR) to genotype *M. tuberculosis complex* (MTBC) is widely used to understand TB transmission and outbreaks. Polymerase chain reaction restriction fragment length

polymorphism analysis (PRA) is preferred because it offers an easy and rapid method to characterize *Mycobacterium* species by applying several targets (16S rDNA, *hsp65*, *IS6110*, and *rpob*) to a single copy of DNA.^{7–9} Currently, mycobacterial interspersed repetitive units–variable number of tandem repeats (MIRU–VNTR) is the reference approach for genotyping MTBC with good specificity and discrimination, which improves the cost-benefit and reduces workload.¹⁰

The application of molecular tests in routine TB diagnosis using slides already prepared for microscopy could be a useful option, especially at sites that are far from reference centers and have poor biosafety infrastructure, making safe sample transport a significant challenge.

For example, a study performed by Carniel et al.¹¹ in the Amazonian area of northern Brazil applied PCR–*IS6110* directly to sputum smears stained with Ziehl–Neelsen (ZN), and they were able to detect TB cases with an accuracy of 91%. This approach offers an affordable strategy for accurate TB diagnosis in remote areas where culture is not available. For these reasons, analyzing DNA extracted from sputum smears offers a good strategy for characterizing *Mycobacterium* species responsible for TB cases in areas with limited laboratory capacity. The objective of this study was to identify possible atypical mycobacterial species that cause TB using a DNA fingerprinting technique on DNA extracted from sputum smear slides.

A retrospective collection of 105 acid-fast sputum smears collected in GYR (November 2012–February 2014) were analyzed. The slide collection corresponded to samples taken at Centro Regional de Salud de la Comarca Guna Yala as part of the TB Control Program of the Ministerio de Salud de Panamá. The 23 Guna Yala (GY) communities included in this study are represented in Figure 1. The ZN method of acid-fast staining was used for detection of acid-fast bacilli (AFB) in smears and examined microscopically. All sputum smears (two slides per sample) were sent to the Tuberculosis Biomarker Research Unit, in the Centro de Biología Celular y Molecular de las Enfermedades laboratory of the INDICASAT-AIP in City of Knowledge.

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The DNA extraction was performed using a method previously described by Van Soolingen et al.¹² Briefly, a slice of the smear was removed and transferred to a microtube containing 1X TE buffer. The material was centrifugated and heated to 90°C for 10 minutes. Cellular lysis was performed with the addition of 40 µL of lysozyme (20 mg/mL) and Proteinase K (10 mg/mL) followed by incubation at 37°C; then the sample was agitated at 65°C. After that, 100 µL of the CTAB was added, and the sample was incubated for 30 minutes. The material was centrifuged at 7,000 rpm for 5 minutes, and phenol-chloroform-isoamyl alcohol (24:24:1) solution was added to the supernatant to remove interfering agents. Absolute ethanol and 3M sodium acetate was used to precipitate the DNA. The extracted DNA was stored at 4°C.

The PRA method based on PCR analysis was performed for the target *hsp65* and *rpob* genes according to the methods described in Lee et al.¹³ Target segments of the *hsp65* and *rpob* genes were amplified by specific primers and viewed following agarose gel electrophoresis. The PCR products were analyzed using the restriction enzymes *Bst*EI, *Hae*III, and *Msp*I. The presence of amplified products was confirmed in 4% agarose gel electrophoresis. The mediate pattern size was compared with a previously described study to confirm NTM species and compared with the international PRASITE database (<http://app.chuv.ch/prasite/index.html>).^{13–15}

Genotyping based on 24-MIRU-VNTR loci was performed to characterize MTBC. The amplification products were analyzed by capillary electrophoresis (Applied Biosystems, Foster City, CA). The number of MIRU-VNTR alleles was determined according to a protocol described by Supply et al.¹⁰

TABLE 1

Characterization of mycobacteria species by *hsp65-rpob* restriction fragment length polymorphism methods

Bacilloscopy	N (%)
1+	2 (1.9)
2+	2 (1.9)
3+	5 (4.8)
AFB positive	17 (16.1)
AFB negative	79 (75.2)
Species ID	
<i>M. tuberculosis</i> Complex	19 (18.0)
Nontuberculosis	7 (6.7)
<i>M. avium</i> complex	4 (3.8)
<i>M. haemophilum</i>	2 (1.9)
<i>M. tusciae</i>	1 (0.9)
No amplification product	79 (75.2)

AFB = acid-fast bacillus.

Among the communities included in this study, Playon Chico (31.5%), Ustupu (13.5%), Nargana (7.9%), Gardi Sugdup (6.7%), and Soledad Mandiga (6.7%) provided the most AFB sputum slides.

A total of 26 sputum smear slides were characterized as *Mycobacterium* spp.; 19 were identified as MTBC, and seven were identified as NTM species (Table 1). The main NTM species were characterized as *M. avium* complex ($N = 4$), *M. haemophilum* ($N = 2$), and *M. tusciae* ($N = 1$). Polymerase chain reaction for both *hsp65* and *rpoB* did not result in amplification for 79 (75.2%) DNA samples extracted from the sputum smears. All MTBC ($N = 19$) were genotyped according to MIRU-VNTR and compared with the MIRU-VNTR *plus*

FIGURE 1. Communities in Guna Yala region in Panama with sputum smear slide collected during 2012–2014. Accessibility to the number of samples are indicated in percentages. This figure appears in color at www.ajtmh.org.

TABLE 2
The 24-loci MIRU-VNTR genotyping of *M. tuberculosis* Complex from sputum smear slide

ID	MIRU 02	VNTR 42	VNTR 43	MIRU 04	MIRU 40	MIRU 10	MIRU 16	MIRU 1955	MIRU 20	VNTR QUB11b	VNTR ETR-A	VNTR 46	VNTR 47	VNTR 48	MIRU 23	MIRU 24	MIRU 26	MIRU 27	VNTR 49	MIRU 31	VNTR 52	VNTR QUB-26	VNTR 53	MIRU 39
GY-1	2	2	3	2	3	5	3	3	0	4	3	4	4	2	5	1	5	3	3	2	1	2	3	2
GY-59	2	2	3	2	3	2	3	3	2	4	3	4	4	2	3	1	5	3	2	3	3	7	5	2
GY-82	2	2	3	2	3	3	3	4	2	2	3	4	4	2	5	1	4	3	3	3	4	2	4	2
GY-117	2	4	4	2	3	4	3	2	2	4	2	4	2	2	6	1	3	3	1	3	1	2	3	2
GY-120	2	4	4	2	3	3	3	2	2	4	2	4	2	1	6	1	3	3	1	3	1	2	3	2
H37Rv*	2	2	4	3	1	3	2	2	2	5	3	4	2	3	6	1	3	3	3	3	5	5	2	2

ETR = exact tandem repeats; MIRU-VNTR = mycobacterial interspersed repetitive units-variable number of tandem repeats; QUB = Queens University of Belfast.

* Control, *Mycobacterium tuberculosis* American Type Culture Collection (ATCC) strain H37Rv.

database (<https://www.miru-vntrplus.org/MIRU/index.faces>). Our findings show five genotypes with 24 complete loci; the obtained samples GY-117 and GY-120 showed a change in a single-locus variant (MIRU 10 [4/3] and VNTR 48 [2/1]); the rest of the DNA samples (GY-1, GY-69, and GY-81) had more than four single locus variants or incomplete MIRU-VNTR ($N = 14$) (see Table 2).

We analyzed a conventional method AFB and simple PRA analysis to identify and characterize NTM. Specifically, of the 79 samples with AFB-negative results according to the microscopic test, 12.6% (10/79) were positive with *hsp65-rpoB* restriction fragment length polymorphism. Under usual practices these cultures would have been discarded, but molecular screening alerted us to the presence of mycobacteria (six MTBC and four NTM) in the samples. A previous study by Carniel et al.¹¹ applied a combination method using ZN/PCR and IS6110-PCR. They reported that the ZN/PCR combination identified seven (24.14%) additional cases, whereas IS6110-PCR showed positivity in 26% of samples that were AFB negative. In our analysis, the sensitivity and specificity of AFB staining were 24.7% and 75.2%, respectively, whereas the sensitivity and specificity of the PRA analysis were 61.5% and 87.4%, respectively. The combined ZN/PCR analysis showed a sensitivity of 61.5% and a specificity of 81.3%. The performance of this strategy with sputum samples with low bacillary load has not been determined.

The proportions of NTM and MTBC found in our study were similar to those reported by Zavala et al.¹⁶ In this study, the National Mycobacterial Laboratory in Panama analyzed 4,257 sputum samples collected between 2012 and 2015 to confirm their identification. This study identified 65.3% (2,783/4,257) as *M. tuberculosis* and 18% (769/4,257) as NTM. Among the NTM, *M. avium* complex was the most frequent NTM strain, accounting for 22.3% of the samples. In contrast, our study showed the most frequent NTM isolate was *M. avium* complex in four (15.3%) samples, followed by *M. haemophilum* in two samples (7.6%) and *M. tusciae* in one sample (3.8%). Together these findings highlight the importance of NTM and the clinical significance in patients with suspected TB.

The number of NTM characterized in GYR is consistent with the increasing rates reported worldwide in recent decades. A multicenter epidemiology study (2013–2017) of NTM in Spain described the frequency and diversity of 65.5% NTM and 34.5% MTB species.⁴

For this type of analysis, the quality of the sputum sample significantly influences the performance of AFB and PRA analyses. High-quality sputum samples resulted in an improved AFB or PRA analysis. Low-quality sputum samples represent a challenge in routine diagnosis. Approximately 25% of sputum samples are not adequate for culture, which leads to high variability in results. The PCR analysis is rapid and sensitive but presents two major problems: 1) false-negative reactions caused by inhibitors and false-positive reactions due to contamination with DNA and amplicons and 2) the influence of respiratory tract microbiota on the performance of AFB/PRA analysis.¹⁷ Culture remains the gold standard for laboratory confirmation of NTM infection. Thus, a direct correlation between molecular and culture results from a sputum specimen (not prepared as an AFB smear) is needed to validate this strategy.

We studied a convenience sputum smear sample set. The true NTM proportion among the GYR inhabitants remains to be determined. We hypothesize that the lifestyle in GY could account for the high proportion of NTM among people with tuberculosis. For example, the GY population harbor a closed cultural behavior, and it is difficult to access their traditions. The population prefers to use traditional medicine at home and does not seek medical attention at government or private health centers. This leads to increased infection rates and difficulty in following up patients. In addition, there is only one AFB laboratory in the GYR, which serves people with limited accessibility and frequent interruptions in anti-tuberculosis therapies.¹⁸

GeneXpert was introduced in GY in 2017 by international support from Global Fund to Combat HIV, Tuberculosis and Malaria.¹⁹ This technology is used specifically for MTBC diagnosis and does not identify NTM. Our results indicate that collecting microscopy slides and applying PCR detection and genotyping methods is one option for the GYR. To genotype MTBC, we support the option to save GeneXpert positive cartridges and use the GeneXpert remnants for further MTBC genotyping, as previously reported.^{11,19–21}

The American Thoracic Society and Infectious Diseases Society of America have provided guidelines to assist in the accurate diagnosis of lung disease caused by NTM.²² These microbiologic, radiographic, and clinical criteria are considered equally important and must be met to make a proper NTM diagnosis. Specifically, the American Thoracic Society recommends having positive culture results from at least two separate expectorated sputum samples. We agree that the same criteria should be applied for NTM molecular diagnosis. At least two sputum smear slides should have positive molecular test results before initiating treatment with antibiotics. On the other hand, the presence of NTM in sputum samples indicates a need for attention in the GYR; the high prevalence of TB and poor access to the health system need to be addressed urgently. Because NTM are not reported infections, the extent and prevalence data are limited. However, there is evidence of their increase not only in low-income countries but also in developed countries.⁴

The diagnosis of MTBC or NTM in remote areas presents major challenges. The main difficulties include late arrival to the reference laboratory in Panama City due to inefficient sample transport mechanisms, resulting in lost or deteriorated specimens that cannot be analyzed. Our study shows that we are able to characterize mycobacteria directly from AFB smears and simultaneously genotype *Mycobacterium* species that most affect rural regions in Panama, such as GY. This is particularly important to provide an affordable alternative to other remote areas, including Ngäbe Buglé and Bocas del Toro, where TB rates are double or triple the national average. These areas demonstrate social determinants for TB disease, including high levels of poverty, internal migrants moving from rural to urban areas, and poor access to health services. Sputum slides can be easily transported at room temperature, making their conservation easier. This would facilitate the characterization of NTM within *Mycobacterium* species in a shortened period and represents an alternative where culture remains challenging in this remote area. Furthermore, we

recommend strengthening laboratory diagnosis within the GYR to identify infections with NTM in a timely manner.

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