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Heterogeneity of *Mycobacterium tuberculosis* Strains Circulating in Panama's Western Region

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Abstract. Tuberculosis remains a challenge in both rural and urban areas. Although a majority of countries display a higher burden in urban areas compared with rural areas, Panama continues to report the highest mortality rate in Central America. Urban areas, such as Panama City, report a high tuberculosis burden, whereas Panama's western region, including the provinces of Chiriquí, Bocas del Toro (both semiurban) and Ngäbe-Bugle (rural), show a lower burden. We aimed to identify highly transmitted *Mycobacterium tuberculosis* strains within rural and semiurban settings of Panama's western region during a 3-year period (2017, 2019, 2021). We randomly selected *87 M. tuberculosis* isolates from a biobank from Panama's western region and analyzed them using allele-specific oligonucleotide polymerase chain reaction and 24-mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR). Our results show only 11.7% (10/85) of *M. tuberculosis* strains identified as prevalent A-Beijing, B-Haarlem, or C-LAM Strains. We found a low prevalence of A, B, and C *M. tuberculosis* strains in both rural and semirural settings compared with isolates collected from the Eastern Colon Province. MIRU-VNTR genotyping revealed a high degree of diversity with no clusters with single loci variation of ≥ 2 loci. These results support the notion that tuberculosis prevalence in the rural and semiurban western region of Panama are not due to previously described highly transmitted strains but is influenced instead by other health determinants, including poor health system access and a lack of systematic transmission chain monitoring. For remote rural and semiurban settings, we recommend allocating resources to reinforce efforts to prevent tuberculosis spread.

INTRODUCTION

Tuberculosis (TB) continues to be cited as one of the leading causes of infectious disease deaths worldwide. It is estimated that one-quarter of the world's population is latently infected with *M. tuberculosis*, and 5% to 15% of those infected will develop active TB disease. Nearly 10.6 million new cases are reported annually, and more than 1.6 million people died of TB in 2021 (including HIV-positive patients).[1] Panama reported higher mortality rates (4.6 per 100,000 population) among Central America countries (https://platform.who.int/mortality).[2] Despite local economic development, Panama's health system remains underprepared for taking on TB challenges. Lack of supplies, technology, and diagnostic equipment interrupt adequate TB health care.[3] In 2021, a total of 1,360 TB cases were reported nationwide, with Panama province (urban city) reporting the highest number of TB cases, showed the prevalence of 315 TB cases per 100,000 population. For the same period, 289 TB cases were reported from Chiriqui and Bocas del Toro province (semiurban), and Ngäbe-Bugle (rural region) reported 88 TB cases (https://www.minsa.gob.pa/).

Globally, the prevalence of TB is considerably higher in urban areas than in rural areas.[1] In some countries, however, these statistics are reversed. In developing countries where a large proportion of the population resides in rural areas, the incidence of TB in those areas is greater than or equal to that of large urban concentrations. Some rural provinces or subpopulations may experience heavier TB burdens due to the poor living conditions, limited healthcare access, and working conditions. In such scenarios, bacterial heterogeneity plays a critical role in shaping local TB epidemiology and presents an additional challenge for TB control.[4,5]

Earlier studies have found that low clustering proportions highlight the role of endogenous TB reactivation as a main disease burden driver in rural areas.[6] Moreover, specific lineages, such as W-Beijing strains found in recent rural transmission networks, are highly associated with multidrug resistance (MDR) and Bacillus Calmette–Guérin vaccination. In fact, the TB transmission route appears to be evident among MDR *M. tuberculosis* strains isolated from rural settings.[7] The transmission pattern of MDR-TB presented mainly as sporadic distribution in small groups within rural villages. Thus, MDR strains are a driving factor in recent TB transmission mechanisms. However, healthcare and social venues, including sites of alcohol consumption, worship, and marketplaces, could still favor TB transmission within rural areas.[8]

The Panama western region includes the semiurban provinces of Chiriquí and Bocas del Toro and the rural Ngäbe-Buglé Comarca. During the 2018–2021 period, this region reported more than 1,300 new TB cases, which accounted for one-quarter of the national total. Poverty, low resources, limited employment opportunities, and limited healthcare access result in community members migrating to and from this western region. Such movement allows for the introduction and export of *M. tuberculosis* strains; understanding the genetic structure of these *Mycobacterium* strains is essential for guiding tuberculosis control efforts.[9,10]

Previous studies have described the genetic structure of *M. tuberculosis* isolates in Panama. Between 2002 and 2004, nearly 16% (37/231 isolates) of MDR -TB cases were identified as part of transmission chains in the provinces of

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Panama and Colon.[11] Later, a genomics study identified 44% MDR-TB isolates belonged to the same LAM9-c1 family and were characterized by the same primary resistance to isoniazid, rifampin, and streptomycin.[12] Simultaneously, 21% of drug susceptible *M. tuberculosis* Beijing strains isolated from a single health center were shown to be part of transmission clusters.[13] Two recent studies using mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing showed nearly half of *M. tuberculosis* strains from Panama were clustered in six transmission groups.[14] Four clusters (A–D) comprised seven to14 isolates each, whereas the remaining two clusters were smaller (E, three isolates, and F, two isolates). Most isolates corresponded to lineage 4, and 7.4% corresponded to lineage 2.[14] Additional prospective studies identified Beijing A strain among 25.5% to 40.5% of TB cases in Colon province and least 5% of cases for B y C Strain.[15] Although this evidence from the central and Caribbean regions of Panama indicates a high proportion of Beijing type A strains, the characteristics of the TB strains circulating in Panama's western region remain poorly understood.

In this study, we aimed to genetically characterize *M. tuberculosis* isolates obtained between 2017 and 2021 from both rural and semiurban areas of Panama's western region. We found a low prevalence of *M. tuberculosis* A, B, and C strains and low clustering rates in both rural and semirural settings for the isolates collected over 3 years. Additionally, we found no clusters by 24-locus MIRU-VNTR, indicating high heterogeneity among the strains.

MATERIALS AND METHODS

***Mycobacterium tuberculosis* isolates.** Eighty-seven *M. tuberculosis* strains (only 85 isolates were successfully tested) were obtained from a collection provided by the Hospital Materno Infantil José Domingo de Obaldía located in the city of David, Chiriquí, Panama. This hospital receives most of the TB patients with symptoms and complications from Panama's western region, including the Provinces of Chiriquí and Bocas del Toro (both semiurban areas) and the Indigenous Ngäbe-Buglé comarca (rural; Figure 1). Variations in these rates over the past 11 years are shown in Figure 2. We conducted a retrospective study of *M. tuberculosis* isolates obtained from clinical samples (sputum, pleural liquid, cerebrospinal fluid, and bronchoalveolar lavage samples) collected from patients with symptomatic respiratory disease. These samples of *M. tuberculosis* strains were cultured using BACT/ALERT Culture Media (bioMérieux Marcy-l'Étoile, France) and archived over 3 nonconsecutive years (2017, 2019, and 2021). The collection of strains is part of standard procedure, which includes a diagnostic GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA) test, identification of GenoType MDR plus, including resistance to Rifampicin and/or Isoniazid (Hain LifeScience, Ft. Mill, SC), and storage of isolates in the Microbiology Laboratory of the HMIJDO, David, Chiriqui.

As a comparison, we included an additional *78 M. tuberculosis* isolates collected from Colon (January 2021 to March 2022). Among these isolates, 5.1% were drug resistant (rifampicine), and 94.9% were drug sensitive (GeneXpert MTB/RIF [Cepheid]/GenoType MDRplus [Hain LifeScience]). These samples were chosen following the same selection criteria as the retrospective strain collection.

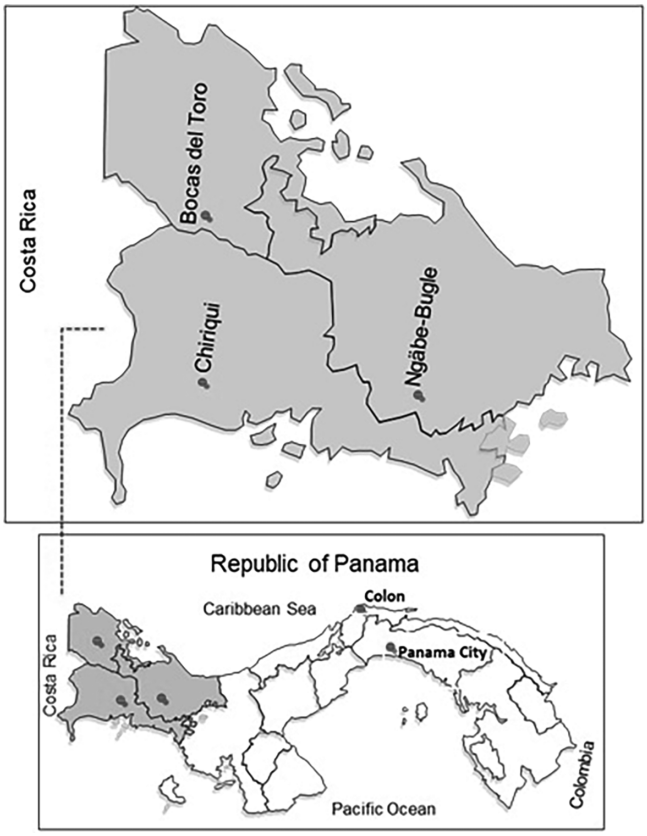

FIGURE 1. Map of Panama's western region: location of Panama's western region includes the provinces of Chiriquí and Bocas del Toro, as well as the indigenous Ngabé-Buglé Comarca. This figure appears in color at www.ajtmh.org.

DNA extraction. DNA extraction was performed using a method previously described by Van Soolingen et al.[19] Briefly, five colonies were transferred to a microtube containing 1× TE buffer. The material was centrifuged and heated to 90°C for 10 minutes. Bacterial lysis was performed with the addition of lysozyme (20 mg/mL) and proteinase K (10 mg/mL) followed by incubation at 37°C; each sample was then shaken vigorously using a vortex and incubated for 10 minutes at 65°C. After that, cetyltrimethylammonium bromide was added and incubated for 30 minutes. The material was centrifuged at 7,000 × *g* for 5 minutes, and a

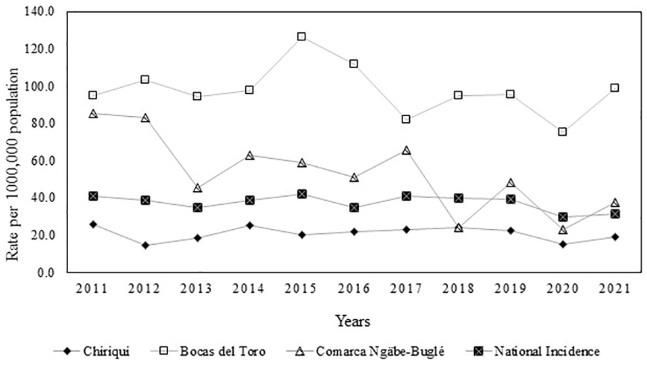

FIGURE 2. Tuberculosis incidence in study population from 2011 to 2021. Source: Health information, Department of epidemiology, Ministry of Health of Panama (https://www.minsa.gob.pa/).

phenol-chloroform-isoamyl alcohol (25:24:1) solution was added to the supernatant to remove interfering agents. Absolute ethanol and 3M sodium acetate were used to precipitate the DNA. The extracted DNA was stored at 4°C for 3 weeks before studies.

Allele-specific oligonucleotide polymerase chain reaction. We conducted specific targeted allele polymerase chain reaction (ASO-PCR) for Beijing *M. tuberculosis* strain A (Beijing A) as previously described.[14] Briefly, 25 μL of the PCR primer pool, including 2, 6, and 5 μM of each ASO-PCR primer, was used. For the B and C Strain primer pools, the concentrations were 2 and 6 μM. The ASO-PCR conditions were as follows: 95°C for 15 minutes, followed by 27 cycles of 95°C for 1 minute, annealing at 64°C (60°C for the B and C strains) for 1 minute and 72°C for 10 minutes. The results were reported as Strain A, no strain A, strain B, no strain B, strain C and no strain C. Strain identities included Strain A (L2, Beijing), Strain B and Strain C (L4, LAM; Figure 3).

Genotyping by MIRU-VNTR. We conducted genotyping on the *85 M. tuberculosis* clinical isolates using single PCRs in-house method based on 24-locus MIRU-VNTR,[20] using custom DNA oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). PCR products were sized using agarose gel electrophoretic analysis and GelAnalyzer Version 19.1. The number of repeats of each locus was assigned, annotated in an Excel database and

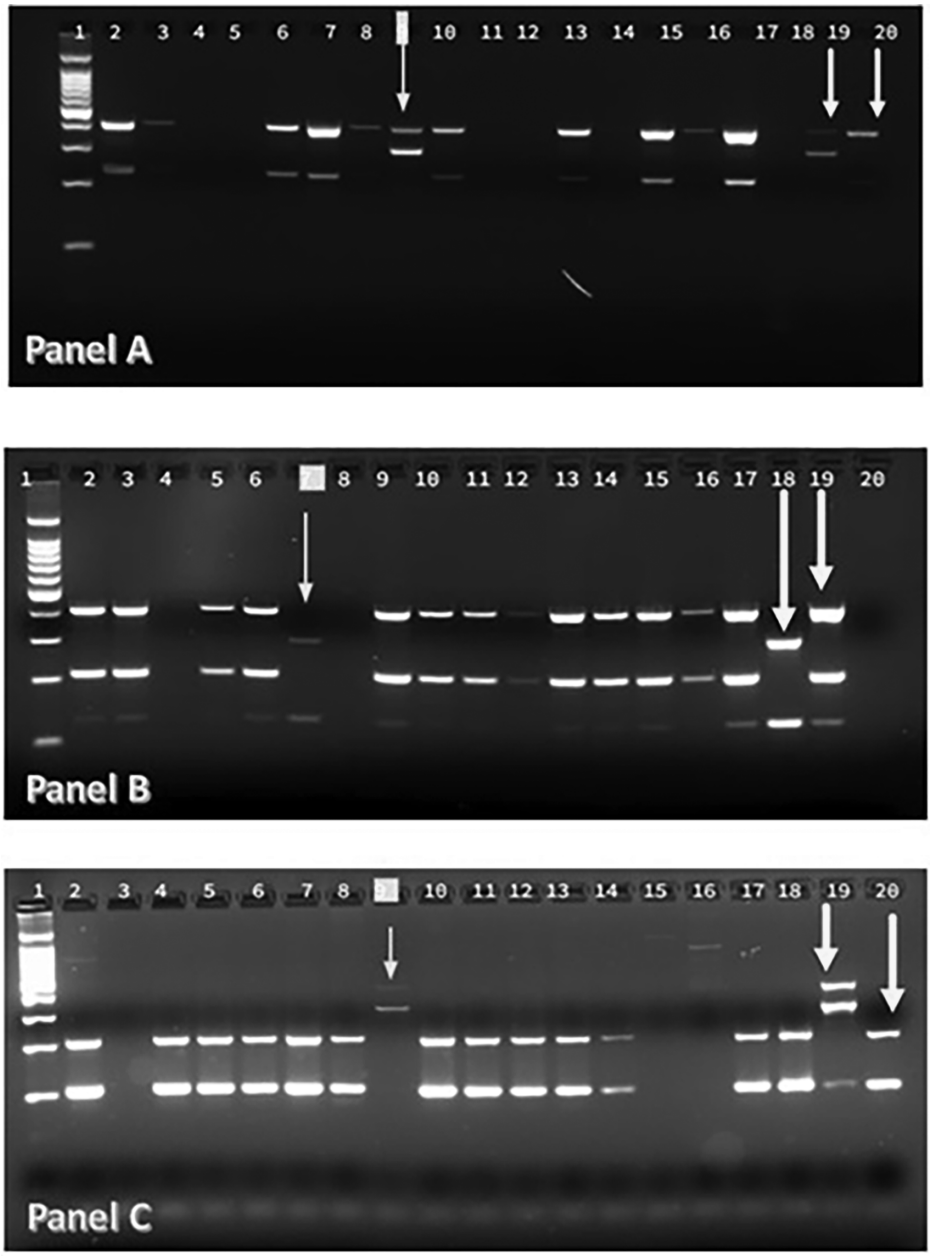

FIGURE 3. Polymerase chain reaction (PCR) products for allele specific oligonucleotide PCRs for *Mycobacterium tuberculosis* A, B, and C strains. Agarose gel electrophoresis (2%) for the identification of the strains. Line 1 represents the molecular weight ladder of 100 bp. (**A**) The arrow in Line 2 indicates the presence of strain A pattern, Lines 19 and 20 as positive controls Strain A, and H37Rv. (**B**) Line 7 Strain B pattern and Lines 18 and 19 as positive controls. (**C**) Line 9 indicates Strain C pattern and respective positive control in the Lines 19 and 20. This figure appears in color at www.ajtmh.org.

then imported into the MIRU-VNTR plus database. We assigned TB lineage and sublineages from the MIRU-VNTR data using a lineage prediction and dendrogram tool from the MIRU-VNTR plus database (https://www.miru-vntrplus.org/MIRU/index.faces).

RESULTS

The bacteria collection was obtained over 3 nonconsecutive years and included 22 strains from 2017, 55 strains from 2019, and eight strains from 2021. These strains represent approximately 8% of the national TB cases for these 3 years according to the Department of Epidemiology of Ministry of Health of Panama (https://www.minsa.gob.pa/). The majority of the collected isolates were from Chiriqui province, representing 68% of the cases. GeneXpert MTB/RIF (Cepheid) and MTBDRsl assays (Hain Lifescience) identified two strains with monoresistance to rifampicin and isoniazid, respectively. On the other hand, only three (3.5%) strains were MDR, and 87.1% were antibiotic sensitive (Table 1). None of the monoresistant or MDR strains were found in the Ngäbe-Buglé Comarca, which highlights that TB burden is due to strains sensitive to antituberculosis drugs.

Next, we analyzed the *85 M. tuberculosis* clinical isolates with ASO-PCR A, B, and C to identify and characterize the A, B, and C strains of lineage 2 and 4, previously described as highly transmitted strains in Panama and Colon Provinces.[14,15] Of the total isolates tested, 10 were classified as prevalent strains, including three (3.5%) strain A (Beijing), two (2.3%) strain B, and five (5.8%) strain C (LAM) (Table 1). We also noted mixed infections with strains A and B (data not shown). Due to the low quality of some DNA samples and the limitations of co-culture, we were only able to analyze *74 M. tuberculosis* isolates using MIRU-VNTR. When we genotyped these isolates, we observed low cluster transmission and a high diversity of genotypes with a single locus variation (SLV) of ≥ 2 with MIRU types represented in dendrograms (Figure 4).

M. tuberculosis strains previously identified as prevalent (strains A, B, and C) were identified in less than 5% of the isolates collected from mixed settings of Chiriquí and Bocas del Toro. When looking the presence of prevalent *M. tuberculosis* strains among semiurban and rural settings, we noted a low representation of the strains (A, B, and C) of the total isolates in Chiriquí and Bocas del Toro, $\leq 5\%$ of the prevalent transmission strains. On the other hand, the A, B, and C strain patterns were not detected among the isolates collected from the rural Ngäbe-Bugle Comarca. We did not find any MIRU-VNTR clusters between western region collection with a difference of ≥ 2 SLVs. When comparing ASO-PCR analysis for 78 prospective *M. tuberculosis* isolates from Colon province (geographically distant location), we noted a different transmission scenario for A, B, and C *M. tuberculosis* strains. We observed transmission clusters in strain A (30.7%), strain B (16.6%), and no cluster for strain C. The remaining 46% (36/78) strains resulted not clustered.

DISCUSSION

The identification of highly transmitted *M. tuberculosis* strains is an important contribution to reinforce TB control efforts. This study aimed to understand the transmission of higher risk *M. tuberculosis* strains and identify the dominant mycobacterial genotypes in Panama's western region. We conducted a retrospective analysis of a 3-year strain collection and searched for the presence of the A Beijing, B

TABLE 1
Summary of study population, drug sensitivity, strain prevalent identification, and genotypes of *Mycobacterium tuberculosis* complex isolates obtained at the Hospital Materno Infantil José Domingo de Obaldía (Chiriqui) of the Occidental region ($N = 85$)

	Isolates, *n* (%)		
Occidental region (province)			
Chiriquí	58 (68.0)		
Comarca Ngäbe-Buglé	12 (14.1)		
Bocas del Toro	9 (10.6)		
No data*	6 (7.0)		
Drug susceptibility			
Susceptible	74 (87.1)		
Isoniazid	1 (1.2)		
Rifampicin	1 (1.2)		
Multidrug-resistant	3 (3.5)		
No data*	6 (7.0)		
Prevalent A, B, and C strains identified by ASO-PCRs and origin isolates			
Strain A (Beijing)	3 (3.5)†		Two from Chiriquí, one from Bocas del Toro
Strain B (Haarlem)	2 (2.3)		One from Chiriquí and no data of rest
Strain C (LAM)	5 (5.8)†		One from Chiriquí and no data of rest
Genotyopes for 74 isolates using MIRU-VNTR analysis			
LAM	37 (50.0)	L4	
Canettii	20 (27.0)		
H37Rv-like	6 (8.1)	L4	
Haarlem	3 (4.0)	L4	
West African 1	2 (2.7)	L6	
S	2 (2.7)	L4	
EAI	2 (2.7)	L1	
Beijing	2 (2.7)	L2	

ASO-PCR = allele-specific oligonucleotide polymerase chain reaction; EAI = East African-Indian; LAM = Latin-American Mediterranean; MIRU-VNTR = mycobacterial interspersed repetitive unit-variable number tandem repeat.
* Data not available.
† Two *M. tuberculosis* isolates were identified as strain mixed.

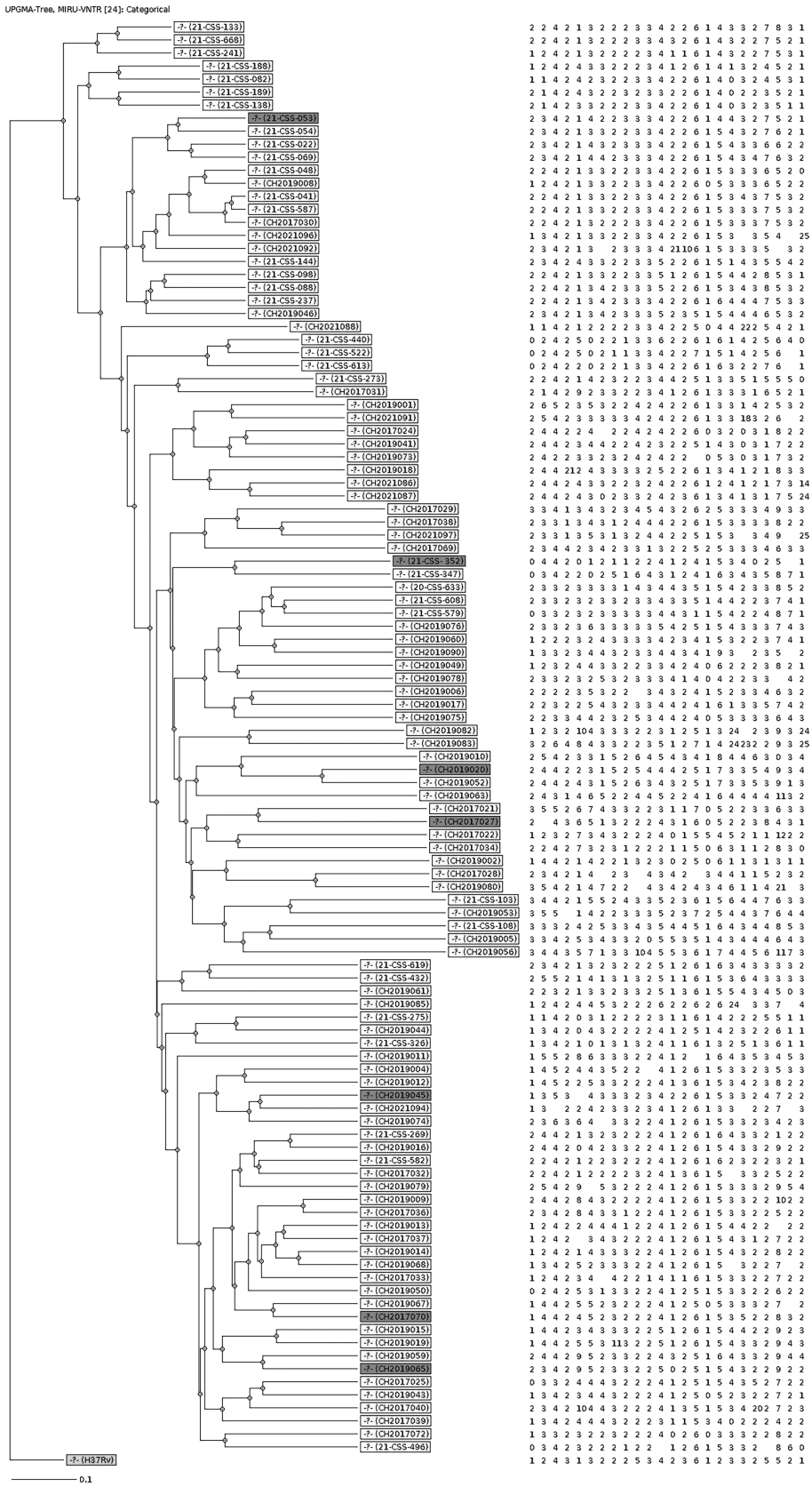

FIGURE 4. Dendrogram obtained with the UPGMA algorithm from mycobacterial interspersed repetitive unit from MIRUVNTRplus. Isolates obtained in western region are highlighted in yellow, in grew comparator isolates from Colón, in green, reference H37Rv. Isolates that showed resistance to rifampicin, isoniazid, or multidrug resistance (MDR) are highlighted in orange; only CH2019065 and 2017070 are MDR. This figure appears in color at www.ajtmh.org.

Haarlem, and C LAM Strains previously identified in Panama and Colon Province.[14,15]

The low clustering rate we found rules out local transmission in the western region of Panama. Instead, these findings support the notion that rural and semiurban settings do not favor TB transmission, and most cases originate from either endogenous reactivation or TB relapse following treatment failure. However, these findings should be interpreted with caution because whole genome sequencing and single nucleotide polymorphism between strains were not analyzed. Moreover, we did not apply the thresholds defined by Walker et al. to establish direct linkage among the isolates.[21] When analyzing the bacterial genetic structure for the entire country, we observe an unequal distribution between the two settings including Colón versus western regions, and remarkably, the Beijing lineage was mainly restricted to Colon.[15] Timely studies of the molecular and genomic epidemiology of tuberculosis can provide a glimpse of high transmission rates for specific strains without a systematic genotypic characterization in the country. This reinforces the need to conduct consecutive analyses with more complete information to strengthen epidemiological surveillance and monitor transmission rates across Panama.

Panama has limited molecular and genomic epidemiological data on the transmission of *M. tuberculosis* strains. The use of epidemiological surveillance tools such as fast allele-specific PCR and ASO-PCR simplifies the steps in identifying high-transmission or higher-risk strains. The province of Chiriquí is characterized by frequent migration because it is the site of several tourist attractions. Apart from this, Chiriqui has regions with low socioeconomic resources and limited healthcare access, making the population susceptible to TB infection and other pathogens of public health importance, such as malaria and HIV/AIDS. We believe the use of ASO-PCR is key for understanding disease transmission in retrospective and prospective studies. Specifically, this strategy can be performed in areas without epidemiological surveillance systems and laboratories with scarce supplies and equipment, such as those located in Chiriquí.

In a previous study, we identified the high-transmission Beijing Lineage *2 M. tuberculosis* strain among 44.1% of isolates from Colon (Panama) using the ASO-PCR for strain A. According to Cerezo-Cortés et al., Beijing genotypes can enter a country, quickly establish themselves, and adapt to the human population to such an extent that the strain co-evolves with the population it infects. Consequently, the genotype can be transmitted over time to neighboring populations.[22] Therefore, the Beijing strain is difficult to control, similar to the Gran Canarias Islands, which reported transmission rate up to $>$ 20% of TB cases over 20 years.[23]

One limitation of our study is the reliance on ASO-PCR to screen for A, B, and C strains. This strategy in Panama's western region allowed us to identify only 3.5%, 2.3%, and 5.8% of the isolates as A, B, and C strains, respectively. There were no historical records or previous reports to delineate the transmission patterns, and the available TB molecular epidemiology studies provide fragmented data insufficient to conduct a chronological country-wide comparison. Previous genotyping studies from our team support the notion of continuous singular and predominant transmission of Beijing Strain A in Colón province, which represents 44.1% of the TB cases.[15] Therefore, additional studies are warranted to provide a detailed description of the prevalent Beijing cluster in Panama. Such an approach would facilitate constructing an *M. tuberculosis* strain entry and distribution timeline across the country.

Although the Beijing lineage is associated with higher prevalence settings, the predominant transmission of a strain in each population is not always associated with this lineage. For example, Argentina reports the active transmission of three MDR strains, and lineage four accounts for 45.1% of the total MDR cases in the country.[24] Similarly, in a study carried out in Zaragoza, Spain, investigating the high percentage of cluster TB cases (46.5%),[25] the predominant strain did not belong to the Beijing lineage (MTZ strain). The authors demonstrated that MTZ strains were responsible for 52.6% of the cases in the cluster.[26]

The ASO-PCR for strains B and C is able to detect the Haarlem and Latin American–Mediterranean strains (linage 4), which are prevalent in the province of Panamá. In a recent study, we showed that B and C strains represented 30% and 19% of strains, respectively, in a transmission cluster.[14] In contrast, the province of Chiriquí demonstrated lower transmission of the B (2.3%) and C (5.8%) strains. Additionally, our MIRU-VNTR analysis showed a high distribution of lineage 4 and a low distribution of lineage 2 (2.7%). Of the samples analyzed, 50% corresponded to LAM, 27% were identified as canettii, and 8% demonstrated H37Rv-like genotypes (Table 1). The circulating bacteria genotypes identified among the incident TB cases in this region suggest that other patterns are driving transmission.

Among Latin American countries, the Haarlem and LAM genotypes belonging to lineage 4 remain the most commonly observed strains. Interestingly, we found the canettii genotype in our collection, which has been described as an ancestor and part of the complex *M. tuberculosis* genotypes.[27] In other settings, canetti's transmission is described as low but with important clinical features.[28,29] Therefore, it would be interesting to deepen the analysis of this genotype in urban and rural settings of Panama's western region. Prospective surveillance could facilitate the analysis and elucidate the chain of transmission as well as evolutionary patterns in the appearance of TB cases.

Transmission surveillance using modern genotyping tools remains useful for understanding and controlling TB, especially in rural and semiurban settings. Previous evidence supports the notion of high bacterial heterogeneity within rural settings playing a critical role in shaping local TB epidemiology within subpopulations.[4] Similarly, our study also shows a high diversity of *M. tuberculosis* strains among both rural and semiurban areas in Panama's western regions. ASO-PCR genotyping enabled us to detect the presence of prevalent strains (A, B, C) in 11.7% of TB cases. We are currently undertaking a systematic collection of all isolates, an approach that will generate the baseline of circulating bacteria and allow us to track highly transmitted strains.

The strategy in our study helps to achieve accurate and cost-effective epidemiological studies, especially in countries with a high TB prevalence.[16] The discriminatory power of MIRU-VNTR typing generally depends on the set of loci used (12, 15, or 24 set), which is the established method for TB molecular surveillance systems in several countries.[17,18]

On the other hand, we believe geographic distance among active cases interferes with disease transmission in rural areas, limiting transmission to susceptible contacts. By using molecular genotyping in surveillance systems, control programs can rapidly identify outbreaks or emerging transmission chains. Consequently, this strategy plays a role in understanding the underlying TB transmission mechanisms in high-burden rural populations.

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