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Short Report: *Mycobacterium tuberculosis* Isolates from Single Outpatient Clinic in Panama City Exhibit Wide Genetic Diversity

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Abstract. Understanding *Mycobacterium tuberculosis* biodiversity and transmission is significant for tuberculosis control. This short report aimed to determine the genetic diversity of *M. tuberculosis* isolates from an outpatient clinic in Panama City. A total of 62 *M. tuberculosis* isolates were genotyped by 12 loci mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) and Spoligotyping. Forty-five (72.6%) of the isolates showed unique MIRU-VNTR genotypes, and 13 (21%) of the isolates were grouped into four clusters. Four isolates showed polyclonal MIRU-VNTR genotypes. The MIRU-VNTR Hunter-Gaston discriminatory index reached 0.988. The Spoligotyping analysis revealed 16 *M. tuberculosis* families, including Latin American-Mediterranean, Harlem, and Beijing. These findings suggest a wide genetic diversity of *M. tuberculosis* isolates at one outpatient clinic. A detailed molecular epidemiology survey is now warranted, especially following second massive immigration for local Panama Canal expansion activities.

Tuberculosis (TB) affects nearly 8.7 million people worldwide.[1] In 2011, most TB cases were reported in Asia (59%) and Africa (26%), although cases were reported to a lesser extent in the Eastern Mediterranean Region (7.7%), the European Region (4.3%), and the Americas Region (3%). Panama stands as the country with the highest TB mortality rate in Central America.[2] In 2012, more than 1,500 TB cases were reported in Panama, for an average incidence rate of 43.1 cases per 100,000 inhabitants.[3] Areas located at the Pacific and Atlantic entries of the Panama Canal have harbored the highest numbers of TB cases since the Canal's construction.[4] Despite sanitation improvements in terminal port cities, recent studies have revealed elevated TB transmission as a result of a high clustering rate among multidrug-resistant TB cases.[5,6] However, data on the transmission of drug-susceptible TB within the general population remain scarce and have not been updated to reflect a second wave of immigration connected with Panama Canal expansion activities.[7]

Mycobacterium tuberculosis genotyping has proven to be the most important laboratory tool in understanding TB transmission.[8] In addition to studies on patient contacts; information on molecular epidemiology is useful for evaluating TB control program results. Genotyping also assists in monitoring molecular markers associated with virulence, immunogenicity, and drug resistance[9]; among the genotyping tools available, the *IS6110*-restriction fragment length polymorphism (RFLP) reference standard method is based on the number of repetitions of the *IS6110* sequence along the *M. tuberculosis* genome.[10] This tool discriminates between clonally related and unrelated isolates. On the other hand, Spoligotyping focuses on detecting 43 spacer sequences in the direct repeat region of the *M. tuberculosis* genome. Unfortunately, the IS*6110*–RFLP method is a complex and laborious procedure, whereas Spoligotyping is faster and simpler but less discriminating.[11,12] The study of mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) is an alternative to genotyping *M. tuberculosis* isolates.[13,14] Our study aimed to characterize the genetic diversity of *M. tuberculosis* isolates in one outpatient clinic using a combination of 12 loci MIRU-VNTR.

A total of 62 clinical isolates were collected at the Social Security Clinical Laboratory of the Complejo Hospitalario Metropolitano Dr. Arnulfo Arias Madrid between January and December of 2005. The strain collection was performed as part of the Panamanian standard of patient care for TB diagnosis and control in Panama City. These isolates accounted for 16.3% of all pulmonary TB cases reported in Panama City in 2005. The DNA extraction was performed using a method described previously.[10] A total of 12 MIRU-VNTR loci were amplified according to a modified protocol described by Cowan and colleagues.[13] The amplification products were analyzed by electrophoresis on an agarose gel. The number of MIRU-VNTR alleles was determined according to the sizes proposed by Cowan and colleagues, which allocate the number of alleles by the fragment size.[13] The allelic diversity for each MIRU-VNTR was calculated using the number of alleles at each locus.[15] The ability to detect the number of allelic repetitions of each allele for every MIRU-VNTR was then classified as high, moderate, or low. We used the Hunter-Gaston discriminatory index (HGDI) to determine the discriminating power of possessing all 12 MIRU -VNTR loci in our study.[16] Spoligotyping was performed on genomic DNA using the standard method described by Kamerbeek and colleagues.[17] The family label and Spoligotype octal code numbers were obtained from the SPOLDB4.0.[18]

Our findings show high genetic diversity of *M. tuberculosis* clinical isolates obtained from outpatients from a clinic in Panama City. Our results reveal that a total of 45 (72.5%) *M. tuberculosis* isolates showed unique MIRU-VNTR patterns (Table 1). Moreover, a total of 13 (20%) isolates were grouped into four clusters. Cluster A (225326-133323) included six isolates, and three isolates were grouped into cluster B

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TABLE 1

MIRU-VNTR genotypes for *M. tuberculosis* clinical isolates with a unique genotype recovered from an outpatient clinic in Panama City (2005)*

Isolate	MIRU-VNTR	Isolate	MIRU-VNTR	Isolate	MIRU-VNTR
1	224325153323	16	224325153221	31	223326153321
2	226425153322	17	224325143324	32	223325173533
3	226226153323	18	224325143324	33	223325153323
4	225335253323	19	224322153323	34	223236253323
5	225335233322	20	224315143324	35	225325153324
6	225326133324	21	224315143323	36	224335253323
7	225325153321	22	224216252321	37	224325153322
8	225325153123	23	223526143322	38	225326133323
9	225325143323	24	223425143324	39	124326154326
10	225325143321	25	223335253324	40	123336253222
11	225226163321	26	223335253321	41	123326153326
12	224425173533	27	223326253321	42	123326163326
13	224336253323	28	223326153321	43	224325153323
14	224335253323	29	223326153321	44	123326153326
15	224326133313	30	223326153321	45	223326153321

*MIRU-VNTR = mycobacterial interspersed repetitive units-variable number of tandem repeats.

(224326-153321). The other two clusters were composed of two *M. tuberculosis* isolates each: cluster C (223325-153324) and cluster D (123237-253227). Four (6.4%) isolates showed only two alleles simultaneously. The presence of two alleles may suggest infection with two or more *M. tuberculosis* strains in these patients. Alternatively, the presence of these strains could be a result of triploid polyclones, similar to that reported for *Mycobacterium bovis* Bacillus Calmette Guérin.[19] These findings indicate the genetic diversity of *M. tuberculosis* circulating in patients with drug-susceptible pulmonary TB in an outpatient clinic in Panama City. Further detailed studies are needed to determine the connection between patients with isolates in the same clusters.

The 12 MIRU-VNTR loci we used showed high discriminatory power for *M. tuberculosis* clinical isolates from the studied outpatient clinic. The discriminatory power of each MIRU facilitated allelic diversity assessment. In our study, the MIRU-VNTR 10, 23, 26, 31, and 40 were highly discriminating. The MIRU 16, 20, and 24 showed low discriminatory power. Thus, our 12 MIRU-VNTR loci showed high discriminatory power, similar to previous reports using this marker set.[14,20] This allelic diversity allowed us to reach an HGDI of 0.988. Thus, we confirmed the discriminatory power of this set of 12 MIRU-VNTR loci for analyzing *M. tuberculosis* isolate samples in Panama City. This feature will be useful in tracking outbreak episodes, relapses, or cross-contamination of *M. tuberculosis* in community-based studies.[21–23] In contrast, the Spoligotyping analysis identified 93% of the clinical *M. tuberculosis* isolates (Table 2). We identified 16 *M. tuberculosis* family Spoligotypes including Latin American-Mediterranean, Harlem, and Beijing. Only four (7%) of the *M. tuberculosis* clinical isolates were not annotated in the SPOLDB4.0 database.

The wide genetic diversity of drug-susceptible *M. tuberculosis* clinical isolates collected from a single outpatient clinic is a limited reflection of population dynamics throughout the Panamanian Isthmus. During the early 20th century, the Panama Canal construction attracted a worldwide workforce, especially laborers from the Caribbean and Europe. As a result, Panama and Colon Cities comprised a wide variety of ethnic backgrounds that possibly harbored various *M. tuberculosis* genotypes. The high diversity of *M. tuberculosis* strains from a single outpatient clinic in our study is one example of this

TABLE 2

Spoligotype family genotypes of *M. tuberculosis* clinical isolates recovered from an outpatient clinic in Panama City (2005)

Octal Spoligotyping code	n (%) of *M. tuberculosis* isolates	Family label
777777777760731	7 (13.0)	T2
776177400000171	6 (11.1)	U (LAM3?)
777777777720771	6 (11.1)	H3
777777607760771	5 (9.3)	LAM9
000076777760671	3 (5.6)	LAM5
704003347760471	3 (5.6)	T4_CEU1
777777777760771	3 (5.6)	T1
000000000003771	2 (3.7)	BEIJING
776177607760771	2 (3.7)	LAM3
777736776000071	2 (3.7)	unknown
777777743760771	2 (3.7)	LAM10_CAM
000000007560771	1 (1.9)	T1
466177400000171	1 (1.9)	unknown
476177400000171	1 (1.9)	unknown
777677607760771	1 (1.9)	LAM9
777736777760771	1 (1.9)	X1
777767777760731	1 (1.9)	T1
777776777760601	1 (1.9)	X2
777777477400001	1 (1.9)	U
777777764020771	1 (1.9)	H1
777777770000000	1 (1.9)	U (likely H)
777777770000171	1 (1.9)	unknown
777777774020731	1 (1.9)	H1
777777774020771	1 (1.9)	H1

hypothesis. A similar effect of migration on *M. tuberculosis* diversity has been shown in other cosmopolitan cities in the Americas.[24,25] These studies have associated the great genetic diversity of *M. tuberculosis* clinical isolates with the mixture of city inhabitants. A century later, Panama is currently expanding the Panama Canal and attracting a new labor force that could introduce new *M. tuberculosis* strains.[7] Detailed surveillance studies using larger data sets are urgently required to monitor and understand the spread of *M. tuberculosis* among Panamanian and immigrant TB case contacts. Such studies would help improve TB control measures to decrease the mortality rate. Prompt genotyping of clinical isolates using state-of-the-art polymerase chain reaction-based tools, such as 24-MIRU-VNTR and Spoligotyping, should be implemented to determine epidemiological relationships and infections with two or more *M. tuberculosis* strains.[26,27] Such a strategy would allow the identification of genotypes that are sustaining the disease burden and provoking death. This approach would also determine if there is any specific *M. tuberculosis* subpopulations related to higher TB transmission within the country.

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