Molecular Characterization of Mycobacterium tuberculosis in Bolivia by 15-Locus MIRU-VNTR Genotyping Reveals Predominance of Haarlem and LAM Lineages

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ABSTRACT

Tuberculosis (TB) remains a major health concern in Bolivia, compounded by limited molecular data on transmission dynamics. This study evaluated the genetic diversity and predominant lineages of 134 consecutive Mycobacterium tuberculosis (MTB) isolates (including susceptible and drug-resistant strains) using a 15-locus MIRU-VNTR genotyping method. We hypothesized that local TB transmission is driven mainly by the reactivation of latent infections rather than the active spread of dominant clones. The 15-locus panel demonstrated high discriminatory power (Hunter–Gaston index = 99.7%), identifying 112 unique profiles (16% clustering). The Haarlem (48%) and Latin American–Mediterranean (LAM) (38%) lineages were predominant, with the Beijing genotype (2.3%) associated with multidrug-resistant (MDR) cases. Analysis via UPGMA dendrograms confirmed considerable MTB genetic heterogeneity in Bolivia, supporting our hypothesis of reactivation over recent transmission. While the 15-locus scheme may slightly underestimate diversity, this study provides essential baseline data to guide molecular surveillance and strengthen national TB control strategies.

Keywords: Mycobacterium tuberculosis; MIRUS-VNTR genotyping; genetic diversity; tuberculosis epidemiology; Bolivia

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Caracterización Molecular de Mycobacterium tuberculosis en Bolivia, mediante genotipificación de 15 locus MIRUS- VNTR, revela predominancia de los linajes Haarlem y LAM

RESUMEN

La tuberculosis (TB) sigue siendo una importante preocupación de salud en Bolivia, agravada por los datos moleculares limitados sobre su dinámica de transmisión. Este estudio evaluó la diversidad genética y los linajes predominantes de 134 aislados consecutivos de Mycobacterium tuberculosis (MTB) (incluyendo cepas susceptibles y resistentes a fármacos) mediante un método de genotipado MIRU-VNTR de 15 locus. Nuestra hipótesis se basó en que la transmisión local de TB está impulsada principalmente por la reactivación de infecciones latentes en lugar de la propagación activa de clones dominantes. El panel de 15 locus demostró una alta capacidad discriminatoria (índice de Hunter–Gaston = 99.7%), identificando 112 perfiles únicos (16% de agrupamiento). Los linajes Haarlem (48%) y Latinoamericano-Mediterráneo (LAM) (38%) fueron los predominantes, con el genotipo Beijing (2.3%) asociado a casos de tuberculosis multirresistente (MDR). El análisis mediante dendrogramas UPGMA confirmó una considerable heterogeneidad genética del MTB en Bolivia, respaldando nuestra hipótesis de la reactivación sobre la transmisión reciente. Si bien el esquema de 15 locus puede subestimar ligeramente la diversidad, este estudio proporciona datos de referencia esenciales para guiar la vigilancia molecular y fortalecer las estrategias nacionales de control de la TB.

Palabras clave: Micobacterium tuberculosis; genotipificación mediante MIRU-VNTR; diversidad genética; epidemiología de la tuberculosis; Bolivia.

INTRODUCCIÓN

Tuberculosis (TB), caused by bacteria of the Mycobacterium tuberculosis (MTB) complex, continues to be a major global public health threat. The World Health Organization (WHO) estimated 10.6 million new TB cases and 1.6 million deaths in 2021, with disruptions from the COVID-19 pandemic reversing years of progress (World Health Organization, 2022). The Region of the Americas, particularly Latin America, faces a persistent and, in some areas, increasing TB burden, exacerbated by factors such as poverty, overcrowding, and healthcare access challenges (Ranzani *et al.*, 2021; Walter *et al.*, 2021).

Bolivia is among the high-TB-burden countries in the Americas, with an estimated incidence of 105 cases per 100,000 population in 2021 (World Data Atlas, 2021). While this represents a decline from previous decades, the rate remains insufficient to meet the WHO's End TB Strategy milestones. A critical component of effective TB control is understanding the pathogen's population structure and transmission dynamics. Molecular genotyping techniques allow for the identification of circulating strains, detection of outbreaks, and differentiation between recent transmission and reactivation of latent infection (Coscolla & Gagneux, 2010).

Among various genotyping methods, MIRU-VNTR has emerged as a gold standard for molecular epidemiological studies of MTB. It offers an optimal balance of high discriminatory power, reproducibility, cost-effectiveness, and data portability for international comparisons (Supply *et al.*, 2006). While previous studies have provided insights into MTB strains in neighboring South American countries (Ritacco *et al.*, 2008; Meza *et al.*, 2014; Balcells *et al.*, 2015), data from Bolivia remain limited.

This study aimed to characterize the genetic diversity and population structure of MTB clinical isolates collected in Bolivia using a 15-locus MIRU-VNTR scheme. The specific objectives were to: (i) determine the discriminatory power of the typing method, (ii) estimate the clustering rate as an indicator of recent transmission, (iii) identify the predominant MTB lineages, and (iv) document the presence of epidemiologically significant strains, such as the Beijing genotype.

MATERIALS AND METHODS

Study Population, Isolate Selection, and Ethical Considerations

As study population, a total of 134 Mycobacterium tuberculosis complex (MTBC) clinical isolates were included in this molecular epidemiological study. These isolates were obtained from the biobank of the National Institute of Health Laboratories (INLASA) in Bolivia, originating from patients with suspected pulmonary tuberculosis from various regions of the country. The time interval between isolate collection (2012-2013) and this publication stems from the research project being conducted during 2018-2019, which required establishing MIRU-VNTR genotyping methodologies previously unavailable in Bolivia.

These historical strains were selected because they represented the only isolates in the INLASA biobank with complete epidemiological metadata essential for robust analysis, unlike more recent isolates that lacked crucial clinical and demographic data. This study provides the first comprehensive molecular baseline of M. tuberculosis in the country, establishing a critical reference point for future research with contemporary strains and maintaining full scientific relevance for informing current tuberculosis control strategies.

The inclusion criteria comprised:

1. confirmed culture-positive for MTBC,
2. availability of accompanying demographic and basic clinical data (e.g., sex, age, treatment history), and
3. availability of a comprehensive drug susceptibility testing (DST) profile.

From the total collection meeting these criteria, 134 isolates were randomly selected using a computer generated random number sequence to ensure a representative sample, encompassing susceptible, monoresistant, and multidrug-resistant (MDR) strains.

On the other hand, the exclusion criteria included: 1) contaminated or non-viable cultures, 2) insufficient biomass for DNA extraction, and 3) isolates identified as non-tuberculous mycobacteria (NTM).

Regarding ethical considerations, this study was approved by the Institutional Review Board of INLASA. As the study utilized anonymized bacterial isolates from the institution's repository, the requirement for individual informed consent was waived.

Drug Susceptibility Testing (DST)

Phenotypic DST for first-line drugs isoniazid (INH) and rifampicin (RMP) was performed on all isolates using the proportion method on Lowenstein-Jensen medium, following WHO standards (World Health Organization, 2022). Mycobacterium tuberculosis H37Rv (ATCC 27294) was used as the quality control strain in each batch of DST. MDR-TB was defined as resistance to at least both INH and RMP.

DNA Extraction and MIRU-VNTR Genotyping

Genomic DNA was extracted from fresh subcultures by means of the PureLink® Genomic DNA Kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration and purity of the extracted DNA were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and DNA was stored at -20°C until further use.

A standardized 15-locus MIRU-VNTR typing scheme was employed for genotyping (Supply *et al*., 2006). PCR amplification was performed in a [Specify thermal cycler model, e.g., Applied Biosystems Veriti®] using the Taq Platinum Hot Start DNA Polymerase Kit (Thermo Fisher Scientific). The 25 µL reaction mixture contained 1X PCR buffer, 2.5 mM MgCl2, 200 µM of each dNTP, 0.2 µM of each primer, 1 U of Taq polymerase, and 50 ng of genomic DNA]. For the QUB-4156 locus, the primers and cycling conditions described by De Beer *et al.* (2014) were used. To ensure PCR quality control, each run included a negative control (nuclease-free water) and a positive control (M. tuberculosis H37Rv DNA).
The PCR products were separated by electrophoresis on 2% agarose gels stained with SYBR™ Gold (Thermo Fisher Scientific) and visualized under UV light to confirm successful amplification before fragment analysis.

Fragment Analysis and Genotype Assignment

The PCR products were sent for fragment analysis. Electropherograms generated by capillary electrophoresis were analyzed with GeneMapper software v4.0 (Applied Biosystems). The number of repeats at each locus was assigned by comparing fragment sizes to published reference tables (Supply, 2005). To ensure accuracy and reproducibility, a random selection of 10% of the samples was genotyped in duplicate, and the results were 100% concordant.

The resulting numerical codes constituted the MIRU-VNTR profile for each isolate. Genotypes were compared, and clustering was defined as two or more isolates sharing an identical 15-locus profile. Unique patterns were defined as profiles observed only once. Lineage assignment was performed by comparing the MIRU-VNTR profiles with the reference database available on the MIRU-VNTR plus website (http://www.miru-vntrplus.org) (Allix-Béguec *et al*., 2008).

Data Analysis

The Hunter-Gaston Discriminatory Index (HGDI) was calculated to evaluate the discriminatory power of each locus and the entire panel (Hunter & Gaston, 1988). A minimum spanning tree (MST) based on the MIRU-VNTR profiles was generated using the MIRU-VNTRplus web application to visualize phylogenetic relationships. Statistical analyses, including the Chisquare test (or Fisher's exact test where appropriate) to assess associations between categorical variables (e.g., drug resistance and clustering), were performed using SPSS Statistics version 15.0. A p-value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

This study provides the first comprehensive molecular epidemiological analysis of M. tuberculosis in Bolivia using a 15-locus MIRU-VNTR scheme. The integrated presentation of results and their discussion allows for a direct and critical interpretation of the findings within their local and global context.

Our analysis revealed a high prevalence of MDR-TB at 21.6% (29/134). Crucially, a strong statistical association was identified between MDR-TB and a history of treatment abandonment or relapse ($p < 0.0001$). The clinical context of the sampled patients is detailed in Figure 1, which shows that a significant proportion of isolates came from patients who had abandoned treatment or were experiencing relapse, underscoring the population where drug resistance is emerging.

This finding transcends a mere statistical correlation; it

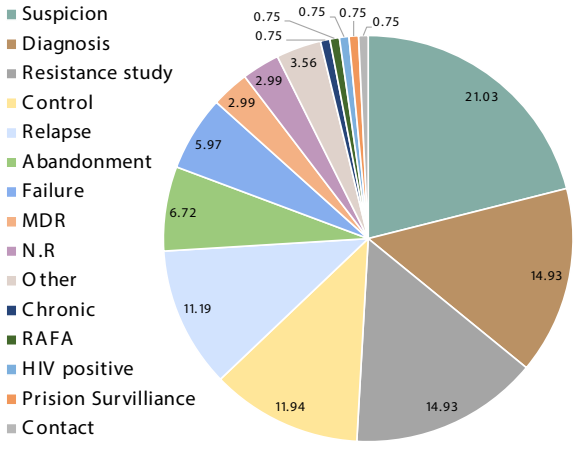

Figure 1. Reasons for taking samples for MTBC isolation in 134 patients in Bolivia (2012-2013).

points directly to a critical vulnerability in the TB control cascade in Bolivia. It indicates that a significant portion of the MDR-TB burden is iatrogenic, likely generated within the healthcare system. This is due to failures in ensuring treatment adherence and completion, rather than being solely due to the primary transmission of resistant strains.

This aligns with broader global concerns that poorly managed TB programs are the main incubators of drug resistance (Bloom & Murray, 1992). The implications for Bolivia are immediate and actionable. While surveillance of drug-resistant strains is essential, our data strongly advocate for a paradigm shift that places equal emphasis on strengthening patient-centered care.

This includes scaling up models like directly observed therapy (DOTS), enhancing psychosocial support, and addressing the socio-economic barriers that lead to treatment abandonment, as highlighted in studies from similar settings in Latin America (Wingfield *et al.*, 2014). Tackling this programmatic gap is not only a clinical imperative but also a cost-effective strategy to curb the generation of new MDR-TB cases.

High Resolution Genotyping Reveals a Transmission Landscape Dominated by Reactivation

Genotyping with the 15-locus MIRU-VNTR panel demonstrated exceptionally high resolution (HGDI = 0.997), distinguishing 112 unique genotypes from the 134 isolates. The discriminatory power of each individual locus is detailed in Table 1, which shows that loci such as QUB-26 (4052) and QUB-11b (2163b) exhibited a high number of alleles, contributing significantly to the panel's overall resolution. Others, like MIRU-04 (580) showed low diversity (h=11).

The resulting genetic profiles formed 11 clusters comprising 22 isolates, resulting in a low clustering rate of 16.4%. The

Table 1.
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15-loci VNTR		HGDI	Size Range Observed	No. of Alleles Observed	Diversity
4052	QUB26	0,83	0-5	6	high
2163b	QUB11b	0,8	1-7	7	high
	QUB415				
4156	6	0,72	0-6	7	high
2996	DIED26	0,71	0-8	9	high
	WONDE				
960	R10	0,71	2-7	6	high
3690	MTub39	0,69	0-10	11	high
802	MIRU40	0,66	1-6	6	high
2165	ETHER	0,65	1-5	5	high
1955	Mtub21	0,64	1-8	8	high
577	ETRC	0,6	3-8	6	moderate
424	Mtub04	0,59	1-5	5	moderate
2401	Mtub30	0,57	1-4	4	moderate
1644	DIED 16	0,47	1-4	4	moderate
3192	MIRU31	0,3	1-5	5	moderate
580	MIRU04	0,11	1-3	3	Low

Use: HGDI represents the allelic diversity value of each locus. VNTR-loci are designated with high, moderate, or low discrimination index based on h >0.6, ≥03 and ≤0.6, and <0.3, respectively.

The distribution of alleles across tehse loci is visualized in Figure 2, demostrating the variability in discriminatory power that contributs to the panel´s overall resolution.

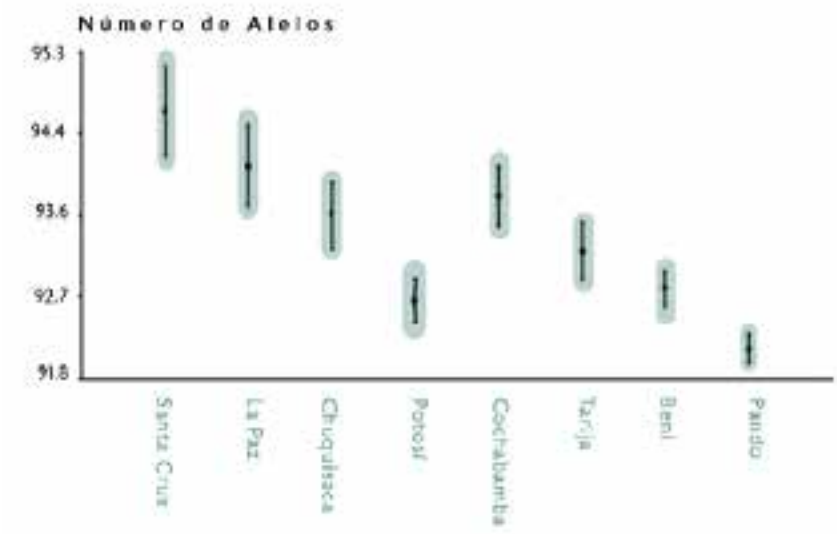

Figure 2. Number of mean alleles by MIRU-VNTRs isolates of M. tuberculosis studied in La Paz, Bolivia by state

phylogenetic relationships between all isolates, illustrating both unique genotypes and clusters, are visualized in the UPGMA dendrogram (Anexo 1)

This pattern, where over 83% of cases have a unique genotype, is classically indicative of an epidemic driven predominantly by the reactivation of latent tuberculosis infection (LTBI) rather than recent, ongoing person-to-person transmission.

This has profound implications for the national TB control strategy. In high-clustering settings, the public health response rightly focuses on aggressive active casefinding and contact tracing to break chains of transmission. However, our results suggest that in Bolivia, a strategy focused only on active TB cases will be insufficient to achieve a decisive decline in incidence. The epidemic is being fueled by a vast and invisible reservoir of latently infected individuals. This evidence provides a powerful scientific justification for the strategic integration of LTBI management into the national program. Prioritizing high-risk groups (e.g., household contacts of active cases, people living with HIV, and other immunocompromised individuals) for LTBI screening and preventive therapy is essential to shrink this reservoir and reduce the long-term incidence of active disease, a strategy that has proven effective in other countries transitioning from high to intermediate TB burden (Dye *et al.*, 2013).

A Unique Phylogeographic Structure: Endemic Diversity and the Threat of Introduced Lineages

The population structure of M. tuberculosis in Bolivia is characterized by a clear predominance of two major lineages, as detailed in Figure 3. The Haarlem (48%) and Latin American–Mediterranean (LAM, 38%) families together account for the vast majority of assigned lineages. A significant proportion of strains (26.9%) were classified as "orphan" genotypes, as they could not be assigned to any known lineage in the MIRU-VNTR plus database.

This high genetic diversity, particularly the abundance of unassigned strains, suggests a long-established and complex evolution of the pathogen in the region, potentially including pre-Columbian strains that are poorly represented in global databases (Bos *et al.*, 2014). This finding is critical for the Bolivian laboratory system, as it implies that standard genotyping tools may lack resolution for a quarter of local strains, potentially leading to misclassification. Future efforts should incorporate whole-genome sequencing (WGS) to fully characterize this unique genetic diversity and understand its clinical and epidemiological significance.

The predominance of the Haarlem lineage offers a

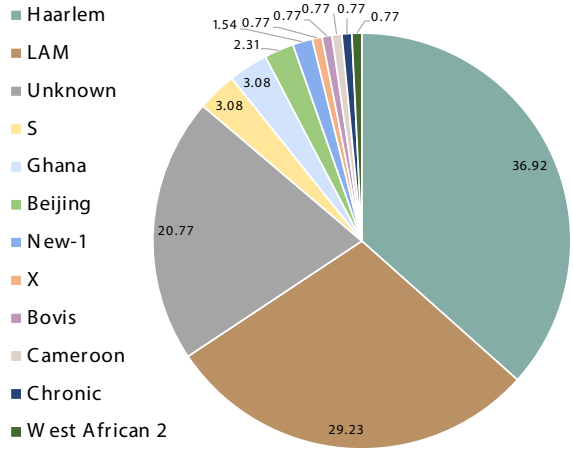

Figure 3. Distribution of lines according to MIRU-VNTR plus from a total of 130 strains of M. tuberculosis isolated in Bolivia from 2012 to 2013

contrasting profile to some neighboring countries like Chile, Peru, and Argentina, where the LAM lineage often dominates (Balcells *et al.*, 2015; Ritacco *et al.*, 2008). This distinct phylogeographic pattern underscores Bolivia's unique epidemiological landscape and highlights the need for tailored control strategies rather than a one-size-fits-all regional approach.

Furthermore, the detection of the Beijing genotype (2.3%), albeit at a low frequency, is a finding of high concern. This lineage is notorious for its association with increased transmissibility, virulence, and outbreaks of MDR-TB worldwide (Parwati *et al.*, 2010). Its emergence in South America has been linked to severe outbreaks, as documented in Buenos Aires, Argentina (Morcillo *et al.*, 2005). Its presence in Bolivia signals its importation and establishes a potential beachhead. This requires constant molecular surveillance and immediate, targeted public health interventions in areas where these cases have been detected, in order to prevent their establishment and spread, which could seriously undermine progress in tuberculosis control.

CONCLUSIONS AND RECOMMENDATIONS

This study confirms that the population structure of Mycobacterium tuberculosis in Bolivia is characterized by high genetic diversity and a low recent transmission rate, as evidenced by the low clustering (16.4%) found using the 15-locus MIRU-VNTR method. The clear predominance of the Haarlem and LAM lineages establishes a distinct phylogeographic profile for the country, while the detection of the Beijing genotype, though rare, signals the introduction of a lineage with high epidemic potential. These findings collectively validate the high resolution and utility of MIRU-VNTR genotyping for ongoing epidemiological surveillance in Bolivia, as established in foundational studies (Supply *et al.*, 2006).

Beyond molecular characterization, our results provide critical, actionable insights for public health policy. The strong association between MDR-TB and treatment history underscores that strengthening patient support and treatment adherence programs is not merely a clinical concern but a fundamental strategy to curb the generation of new drug-resistant cases. Furthermore, the epidemic pattern dominated by reactivation provides a compelling evidence base for the Bolivian TB program to strategically integrate the management of latent tuberculosis infection (LTBI) into its national control strategy, targeting high-risk groups to reduce the future burden of disease. In this regard, the following is recommended:

Implications for Local Health Policy and Specific Recommendations for Bolivia

The findings from this study translate into specific, actionable recommendations for the Bolivian TB control program:

- **Strengthen the Patient Support Cascade**. The National TB Program should prioritize interventions that reduce treatment abandonment, a key driver of MDR-TB. This includes implementing and funding robust patient support systems, such as digital adherence technologies, nutritional support, and transportation subsidies, which have shown success in improving treatment outcomes in resource-limited settings (Arinaminpathy *et al.*, 2022).
- **Develop a National LTBI Management Plan.** Given the evidence that reactivation is a major driver of the epidemic, the program should develop and pilot a phased national plan for the management of LTBI, starting with the systematic screening and preventive treatment of household contacts of bacteriologically confirmed pulmonary TB patients, as recommended by the WHO (World Health Organization, 2018).
- **Enhance Molecular Surveillance.** The detection of the Beijing genotype necessitates an active surveillance response. We recommend establishing a sentinel surveillance system in the regions where these strains were found, with routine genotyping of all MDR-TB isolates to monitor for any clustering that would indicate local transmission of this worrisome lineage.
- **Build Genomic Capacity.** The high rate of orphan genotypes underscores the limitation of current databases and methods. Bolivia should invest in building national capacity for Whole Genome Sequencing (WGS), which provides superior resolution for outbreak investigation, drug resistance prediction, and characterizing the unique diversity of circulating strains (Walker *et al.*, 2022).

Limitations of the study and future research

While this study provides crucial baseline data, its interpretations must be considered within its limitations. First, the use of archived isolates from a two-year period (2012-2013) may not fully represent the current molecular epidemiology of TB in Bolivia, and a prospective, nationwide study is warranted to confirm these findings. Second, the 15-locus MIRU-VNTR scheme, while highly discriminatory, has lower resolution than WGS for confirming recent transmission within clusters and for comprehensively characterizing the orphan genotypes identified. Finally, the lack of detailed patient-level data (e.g., HIV status, precise geographic location within Bolivia) limited our ability to perform more nuanced risk factor analyses for specific lineages or clustering.

These limitations directly inform the trajectory of future research. Prospective, nationwide studies incorporating detailed patient metadata and WGS are essential to build upon this baseline. WGS will be particularly critical for elucidating the nature of the orphan genotypes, confirming Beijing lineage transmission chains, and providing comprehensive drug resistance profiles. By addressing these gaps, future work can further refine TB control strategies, ensuring they are precisely tailored to the evolving epidemiological landscape of Bolivia, ultimately contributing to the global goal of TB elimination.

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Anexo 1. UPGMA dendrogram showing the phylogenetic links among 130 isolates from Bolivia (2012-2013)