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Review

Genotyping methods and their contributions to the study of tuberculosis dynamic in Latin America

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Abstract

Introduction: *Mycobacterium tuberculosis* genotyping has impacted evolutionary studies worldwide. Nonetheless, its application and the knowledge generated depend on the genetic marker evaluated and the detection technologies that have evolved over the years. Here we describe the timeline of main genotypic methods related to *M. tuberculosis* in Latin America and the main findings obtained.

Methodology: Systematic searches through the PubMed database were performed from 1993 to May 2021. A total of 345 articles met the inclusion criteria and were selected.

Results: Spacer oligonucleotide typing (spoligotyping) was the most widely used method in Latin America, with decreasing use in parallel with increasing use of mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) and whole genome sequencing (WGS). Among the countries, Brazil, Mexico, and Argentina had the most publications, and a considerable part of the articles were in collaboration with Latin American or non-Latin American institutions; a small proportion of studies needed partnerships to perform the genotypic methods. The genotypic methods allowed the identification of *M. tuberculosis* genotypes with greater capacity for clonal expansion and revealed the predominance of the Euro-American lineage in Latin America. There was a notable presence of the Beijing family in Peru and Colombia.

Conclusions: The data obtained demonstrated the importance of expanding collaborative networks of tuberculosis (TB) research groups to countries with low productivity in this area, the commitment of the few Latin American countries to advance TB research, as well as the inestimable value of building a Latin America database, considering ease of population mobility between countries.

Key words: genotyping; IS*6110*-RFLP; spoligotyping; MIRU-VNTR; WGS; *Mycobacterium tuberculosis*.

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Introduction

Despite the global efforts to control the dissemination of *Mycobacterium tuberculosis*, 9.9 million new cases of tuberculosis (TB) were estimated in 2020. Of these, only 5.8 million were reported, representing an 18% reduction in the number of reported TB cases, when compared with 2019 (7.1 million). This scenario was related with the coronavirus disease 2019 (COVID-19) pandemic. It is important to note that around 3% of the TB cases were in the Americas, which includes Latin America, mostly composed of lower middle and upper middle income developing countries [1,2].

Seeking to equalize TB incidence and mortality on a global level to that seen in high-income countries, the World Health Organization (WHO) has developed the end TB strategy supported by three pillars. The goal set in the third pillar is to intensify research and innovation for TB control. Increase in the use of molecular tools could improve the understanding of the epidemiological and evolutionary characteristics of *M. tuberculosis* in each country, thereby enabling the discovery of new tools while informing public health policies, interventions, and strategies that are appropriate to each socioeconomic context [3,4].

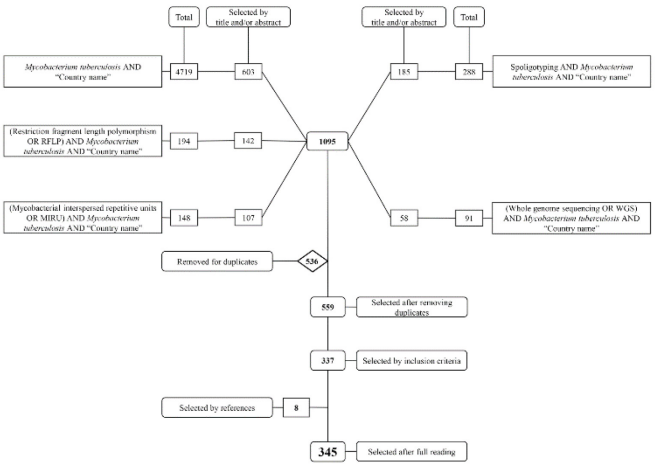
Genotypic methods have improved the understanding of the dynamics of transmission of several pathogens, especially during outbreaks and epidemics (e. g. [5,6]). However, since *M. tuberculosis* is a genetically conserved microorganism, the genotypic methods used in the 1980s were not very useful for this bacterium [7,8]. This scenario changed with the development and standardization of the IS*6110*-restriction fragment length polymorphism

(IS*6110*-RFLP) method in the 1990s [8], as well as the spacer oligonucleotide typing (spoligotyping) [9] and the mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) methods [10,11]. Currently, whole genome sequencing (WGS) using next-generation sequencing (NGS) technologies allows for a more complete understanding of this pathogen's population structure while providing the ultimate discriminatory power [12–14].

The IS*6110*-RFLP method has a high discriminatory capacity and reproducibility; however, due to its need for a large amount of DNA with a high degree of purity and integrity (2-3 µg), difficult portability and because it is more laborious it has been progressively replaced by polymerase chain reaction (PCR)-based methods [14,15]. Spoligotyping has low discriminatory power compared to IS*6110*-RFLP, since it evaluates polymorphisms at a single direct-repeat locus, overestimating clonal transmissions, which limits its application as a molecular epidemiology tool. Nevertheless, Spoligotyping remains useful for discriminating genetic signatures and identifying phylogeographic distributions through specific spacer patterns [9,14,16].

MIRU-VNTR has integrated the possibility of studying *M. tuberculosis* both epidemiologically and phylogenetically, and it is currently considered the gold standard method, especially in lower middle and upper middle income developing countries, such as the Latin American countries. This is because it is highly reproducible. It is also as discriminatory as IS*6110*-RFLP but more portable, and allows classification up to subfamily level, similar to spoligotyping [11,14,15,17]. Nonetheless, in depth understanding of a genotype and inference on the directionality of transmission is only possible by WGS-NGS, which detects genetic changes resulting from single nucleotide polymorphisms (SNP), insertions, and deletions, allowing accurate identification of genetic signatures and phylogenetic relationships. Although the time, cost, and complexity of WGS-NGS methodologies have been substantially reduced, their use is still limited to a few laboratories [12-14,18].

Figure 1. Flowchart of the literature review methodology.



Even though the aforementioned genotyping methods are extremely useful for TB epidemiology, it should be noted that they were established and standardized in socioeconomically developed countries with lower TB incidence [8–12]. Applying these methods in regions with high TB incidence enabled their validation on a large scale, and contributed to the phylogeographic understanding of *M. tuberculosis* strains circulating in Latin America [11,19,20].

The objectives of this systematic literature review were (1) to investigate the usage trend of the conventional genotypic methods and WGS in Latin America; (2) to describe the distribution of studies among Latin American countries; (3) to evaluate the frequency of collaborations with Latin American and non-Latin American institutions and; (4) to present the most notable *M. tuberculosis* related contributions of genotypic methods in the pre- and post-WGS eras in Latin America.

Methodology

The articles selected for this study were published between 1993 (year of publication of the IS*6110*-RFLP method seminal article [8]) and May 2021 and recovered through the PubMed electronic platform. Searches were performed for all Latin American countries using the descriptors: *Mycobacterium tuberculosis* AND "(Restriction fragment length polymorphism OR RFLP)" / "Spoligotyping" / "(Mycobacterial interspersed repetitive OR MIRU)" / "(Whole genome sequencing OR WGS)" AND "Argentina" / "Bolivia" / "(Brasil OR Brazil)" / "Chile"/"Colombia" / "Costa Rica" / "Cuba"/"Ecuador" / "El Salvador" / "Guatemala" / "Haiti" / "Honduras" / "Mexico" / "Nicaragua" / "Panama" / "Paraguay" / "Peru" / "(República Dominicana OR Dominican Republic)" / "Uruguay" / "Venezuela".

The articles were reviewed by looking at the titles and/or abstracts, analyzing the methodologies to verify the inclusion criteria, and subsequently by reading the full texts. Only articles that met the following inclusion criteria were included in the final selection:

- Used IS*6110*-RFLP, spoligotyping, MIRU-VNTR 12-, 15- and/or 24-*loci* and/or WGS methods; Used

isolates from the country of the search: Latin American countries have been defined as American countries that share historical characteristics of colonization and speak romance languages with Latin roots (Spanish, Portuguese, and French).

- Was not a review article.

The searches for the Latin American countries resulted in 1,095 articles selected from the titles and/or abstracts. After removing duplicates and following the inclusion criteria, 337 articles met the eligibility criteria and were read in full (Figure 1). After reading the full text, additional eight articles were included through the references of the selected articles, to reach a total of 345 articles.

After selecting the 345 articles, the genotypic methods used and the number of articles published per year were tabulated to investigate the trend in the use of genotypic methods and to describe the distribution of studies in Latin American countries. The MIRU-VNTR method, at times, was stratified due to its discriminatory power and applicability variations: 12-*loci* MIRU-VNTR has low discriminatory power and was used in epidemiological studies; 15- and 24-*loci* MIRU-VNTR (presented together throughout the article) have high discriminatory power and were used in epidemiological and phylogenetic studies.

Collaborations with Latin American and/or non-Latin American institutions were assessed by the authors institutional affiliations, mentioned in the articles. In addition, complementary analyses in the *Biblioteca Virtual em Saúde* (BVS) platform, which indexes Latin American articles, were performed, since this review used only the PubMed electronic platform, the major international database in biomedicine. Finally, the main contributions of the genotypic methods for the TB dynamic in Latin America were presented.

Table 1. Articles that used individually or in combined genotypic methods in Latin America between 1993 and 2021.

Genotypic method	Number of articles N = 345, (%)
Spoligotyping	58 (16.8)
Spoligotyping + IS*6110*-RFLP	58 (16.8)
Spoligotyping + MIRU-VNTR	62 (18.0)
Spoligotyping + WGS	9 (2.6)
Spoligotyping + IS*6110*-RFLP + MIRU-VNTR	14 (4.1)
Spoligotyping + IS*6110*-RFLP + WGS	1 (0.3)
Spoligotyping + MIRU-VNTR + WGS	8 (2.3)
IS*6110*-RFLP	61 (17.7)
IS*6110*-RFLP + MIRU-VNTR	2 (0.6)
IS*6110*-RFLP + WGS	1 (0.3)
MIRU-VNTR	27 (7.8)
MIRU-VNTR + WGS	16 (4.6)
WGS	28 (8.1)

IS6110-RFLP: IS*6110*-Restriction Fragment Length Polymorphism; MIRU-VNTR: Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat; Spoligotyping: Spacer Oligonucleotide typing; WGS: Whole Genome Sequencing.

Results

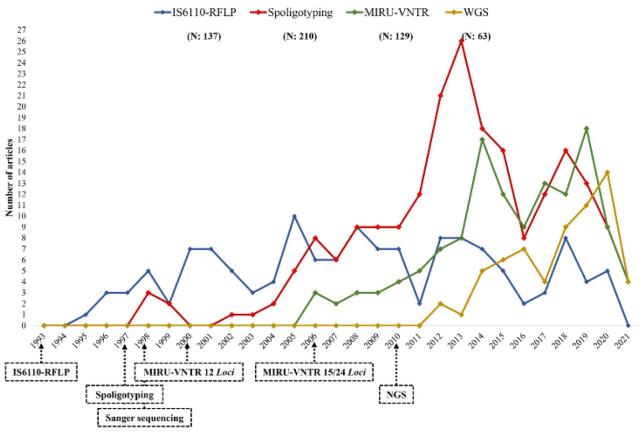
Evolution of the use of genotypic methods in Latin America

In molecular epidemiology, many authors use a combination of at least two genotypic methods to obtain more robust and accurate results, to achieve greater discriminatory power, and to compare different methods [21]. Considering this, 49.6% (171/345) of the articles analyzed used two or three genotypic methods simultaneously. For this reason, the sum of articles presented below for each method exceeds the total number of articles selected (345 articles).

Figure 2 shows the overall use of IS*6110*-RFLP, spoligotyping, MIRU-VNTR and WGS in Latin America through the number of articles published between 1993 and May 2021. Spoligotyping was the most documented method in Latin America, reported in 60.9% (210/345) of the articles reviewed. The second most used method was IS*6110*-RFLP in 39.7% (137/345) of articles, followed by MIRU-VNTR in 37.4% (129/345), and WGS in 18.3% (63/345).

Since the publication of the seminal article on spoligotyping in 1997 [9], its use increased rapidly, averaging 8.6 articles per year, and it continued to be

Figure 2. Use of genotypic methods in Latin America over time.



Each line represents the evolution of one of the methods through the number of articles published with that method (y axis). At the bottom of the image are highlighted the years seminal articles were published on each method. NGS: Next-Generation Sequencing; IS*6110*-RFLP: IS*6110*-Restriction Fragment Length Polymorphism; MIRU-VNTR: Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat; WGS: Whole Genome Sequencing; Spoligotyping: Spacer Oligonucleotide typing.

used even though new methods had been standardized. However, its use decreased progressively after 2014, following the increase in the use of MIRU-VNTR and WGS (Figure 2). The frequent use of spoligotyping was due in part because results from this method were easily interpreted, and due to the development of the international database, SITVIT2 [16]. This database allowed researchers to monitor the global distribution of genotypes and perform interlaboratory comparisons [16].

Spoligotyping was the second most used method individually, reported in 16.8% of the articles analyzed (58/345). Nonetheless, its limited discriminatory power warranted its use in combination with other genotyping methods [15]. Thus, we observed that spoligotyping was generally performed in combination with MIRU-VNTR or IS*6110*-RFLP (Table 1).

Although the seminal article on IS*6110*-RFLP was published in 1993 [8], there had been little variation in the number of articles using this method per year in Latin America, averaging 4.9 articles per year since its standardization (Figure 2). This may be because this method is laborious and requires high amount of purified DNA. On the other hand, the high discriminatory power can explain why IS*6110*-RFLP was the most used method alone [14, 15], present in 17.7% (61/345) of the articles analyzed (Table 1).

Regarding MIRU-VNTR, there were differences in the use of this method in the 12-*loci* format, standardized in 2000 [10], and in the 15- or 24-*loci* formats, standardized in 2006 [11]. Publications with MIRU-VNTR 12-*loci* varied little over the years, and it was rarely used alone (4/129) due to its low discriminatory power when compared to the IS*6110*-RFLP method [15]. However, the use of MIRU-VNTR 15- or 24-*loci* had increased over the years, averaging 5.9 articles per year (Figure 2). In addition, the 15- or 24-*loci* formats were used alone in 17.8% (23/129) of the articles that used this method. This was mainly because the 15- or 24-*loci* formats had high discriminatory power and can generally correlate with the spoligotyping genetic signatures [17].

MIRU-VNTR was frequently used in combination with other methods (102/129), mainly spoligotyping (62/129) (Table 1), to discriminate spoligotyping-designated genotypes and enable more robust phylogenetic relationship analyses. The international online database, MIRU-VNTRplus [22], was also noteworthy, allowing genotype comparisons, and analysis of both phylogenetic relationships and genotypes geospatial distribution [11,22].

Although the number of publications with WGS was low in Latin America (63/345), its use had been increasing rapidly since 2012 (Figure 2). WGS was used alone in 44.4% (28/63) of the articles containing this approach (Table 1). Nevertheless, it was often employed to provide additional resolution to the genetic profiles detected by spoligotyping and MIRU-VNTR and/or in studies with a limited number of strains.

The small number of publications using WGS could be explained by the excessive cost of sequencing and the limited number of professionals trained to analyze the results. However, the reduction in sequencing cost and the development of simplified data analysis platforms made WGS accessible for more accurate molecular and evolutionary epidemiological analyses

Table 2. Scientific production listed in PubMed on *Mycobacterium tuberculosis* genotyping in Latin American countries (1993–2021).

Country	TB new cases*	TB incidence (100,000 pop)*	Articles N (%)	Articles with partnerships for the genotyping execution N (%)	Articles with Latin American partnerships N (%)	Articles with non-Latin America partnerships N (%)
Argentina	13,000	29	45 (13.0)	4 (1.2)	7 (2.0)	14 (4.1)
Bolivia	12,000	106	1 (0.3)	1 (0.3)	1 (0.3)	1 (0.3)
Brazil	96,000	46	128 (37.1)	14 (4.1)	4 (1.2)	66 (19.1)
Chile	3,300	18	8 (2.3)	1 (0.3)	2 (0.6)	2 (0.6)
Colombia	18,000	35	37 (10.7)	10 (2.9)	5 (1.4)	18 (5.2)
Costa Rica	510	10	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cuba	730	6,5	7 (2.0)	0 (0.0)	4 (1.2)	4 (1.2)
Dominican Republic	4,500	42	1 (0.3)	1 (0.3)	1 (0.3)	0 (0.0)
Ecuador	7,900	46	7 (2.0)	1 (0.3)	3 (0.9)	1 (0.3)
El Salvador	3,800	58	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Guatemala	4,600	26	2 (0.6)	2 (0.6)	0 (0.0)	2 (0.6)
Haiti	19,000	170	6 (1.7)	6 (1.7)	0 (0.0)	5 (1.4)
Honduras	3,000	31	5 (1.4)	4 (1.2)	0 (0.0)	3 (0.9)
Mexico	30,000	23	52 (15.1)	9 (2.6)	1 (0.3)	27 (7.8)
Nicaragua	2,800	43	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Panama	1,600	37	7 (2.0)	4 (1.2)	0 (0.0)	5 (1.4)
Paraguay	3,300	46	3 (0.9)	0 (0.0)	3 (0.9)	1 (0.3)
Peru	39,000	119	45 (13.0)	18 (5.2)	5 (1.4)	32 (9.3)
Uruguay	1,200	35	5 (1.4)	0 (0.0)	1 (0.3)	2 (0.6)
Venezuela	13,000	45	15 (4.3)	3 (0.9)	5 (1.4)	5 (1.4)

***** Estimates of TB burden for 2019. Source: https://worldhealthorg.shinyapps.io/tb_profiles.

[13,14,23,24]. This was illustrated by the gradual increase in the use of WGS use over time, peaking in 2020 (14/63).

Large variations were observed while analyzing the specific contributions of each country. Only five countries (Brazil [39,9%], Mexico [22%], Argentina [10.6%], Peru [7.6%] and Colombia [7.3%]) contributed strongly over the years with approximately 90% of the articles analyzed. The greater number of studies coming from these countries is corroborated by the trend found by Torres-Pascual *et al.* (37.1%; 15.1%; 13%; 13%; and 10.7%, respectively) [25], despite the difference between the periods established for the searches. The contribution of other countries ranged from 0% to 4.3%.

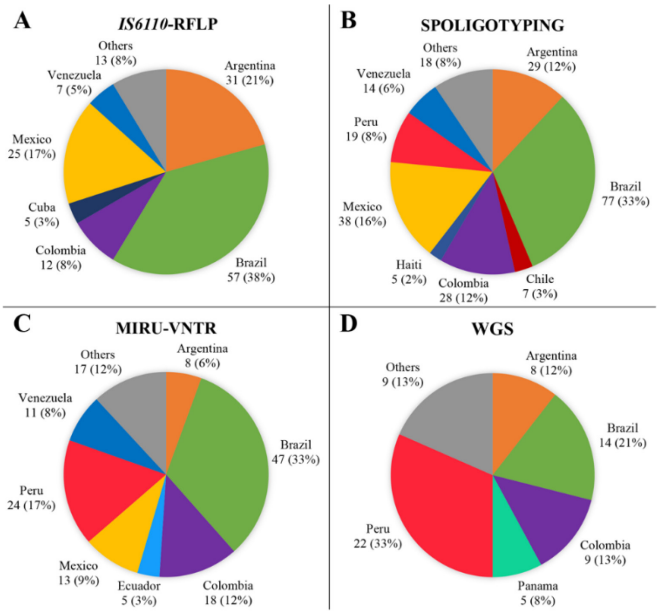
The countries with the highest representation of articles in this study, regardless of genotypic method, also had the highest estimates of TB incidence in Latin America. Together, Brazil, Mexico, and Peru account for more than 50% of the estimated TB burden for the Americas. The other countries, which have high (above 45 cases/100,000 population) and intermediate (between 45 and 10 cases/100,000 population) TB incidence rates, as well as Costa Rica and Cuba, which have low TB incidence rates (below 10 cases/100,000 population) (Table 2) [2], had few or no articles selected.

To verify if the scarcity of articles could be associated with a limitation of this study, namely, the use of only one database, additional searches were performed on the BVS platform. Nevertheless, no articles using the genotypic methods analyzed were retrieved, except for three from Cuba and one from Haiti. Taken together, these data showed the low contribution of most Latin American countries to *M. tuberculosis* molecular research. There may be few research groups focused on epidemiological and evolutionary investigations of *M. tuberculosis* in these countries.

Figure 3 shows the number of articles using IS*6110*-RFLP (A), spoligotyping (B), 12-, 15- or 24-*loci* MIRU-VNTR (C), and WGS (D) in Latin America. Articles using IS*6110*-RFLP were distributed in 12 countries, with more than 75% coming from Brazil, Argentina, and Mexico (Figure 3A). Only eight countries had five or more studies using spoligotyping (Figure 3B), with the largest proportion of these coming from Brazil, Mexico, Argentina, and Colombia. Among the countries that used the MIRU-VNTR method (Figure 3C), only seven had five or more articles, with most concentrated in Brazil, Peru, and Colombia. Regarding WGS (Figure 3D), more than 50% of the articles came from Peru and Brazil. In the other countries, the use of WGS was still scarce, ranging from zero to nine articles. Furthermore, WGS was used as the sole methodology in about 45% of the articles that used it.

When comparing the frequency of methods among countries, Argentina was the only country that more frequently used IS*6110*-RFLP (68.9%; 31/45), mainly in combination with spoligotyping (21/31). Spoligotyping was used most frequently in Brazil (60.2%; 77/128), Colombia (75.7%; 28/37) and Mexico (73%; 38/52) and was used in combination with 12- and 15/24-*loci* MIRU-VNTR in Brazil (34/77), 15/24-*loci* MIRU-VNTR (10/28) in Colombia, and IS*6110*-RFLP (18/38) in Mexico. Meanwhile, in Peru, WGS (48.9%; 22/45) was used most frequently, either alone (45.5%; 10/22) or with 15/24-*loci* MIRU-VNTR (45.5%; 10/22). In the remaining countries, minor variation was

Figure 3. Proportion of articles that used genotypic methods for each Latin American country (1993 to 2021).



Countries that had no articles are not represented. Countries included in the category "other" had 1 to 4 articles. (A) Articles that used the IS*6110*-RFLP method. Others: Chile, Ecuador, Honduras, Paraguay, Peru, and Uruguay. (B) Articles that used the Spoligotyping method. Others: Bolivia, Cuba, Ecuador, Guatemala, Honduras, Panama, Paraguay, and Dominican Republic. (C) Articles that used the 12-, 15- and/or 24 -*loci* MIRU-VNTR methods. Others: Bolivia, Chile, Cuba, Haiti, Panama, Paraguay, Dominican Republic, and Uruguay. (D) Articles that used the WGS. Others: Ecuador, Guatemala, Haiti, Mexico, and Uruguay. IS*6110*-RFLP: IS*6110*-Restriction Fragment Length Polymorphism; MIRU-VNTR: Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat; WGS: Whole Genome Sequencing; Spoligotyping: Spacer Oligonucleotide typing.

seen in the proportion of articles published with each method, separately or in combination.

Approximately 60% (202/345) of the Latin American studies analyzed were conducted in collaboration with other institutions, Latin American and/or non-Latin American. Partnerships with non-Latin American institutions comprised 52.2% of the studies, while partnerships between Latin American institutions comprised only 2.9% of the studies. In addition, a small proportion (3.5%) of the studies established partnerships with both Latin American and non-Latin American institutions. Among the 13 countries participating in Latin American collaborations, Argentina was the country that most frequently collaborated with the other Latin American countries, mainly Brazil and Colombia (Table 2).

Latin American countries had also established collaborations with non-Latin American institutions in 19 countries. The most frequent collaborators were the United States (US; 24.6%), France (11.6%) and the United Kingdom (6.1%). Approximately 85% of the studies conducted with partnerships with non-Latin American institutions came from Brazil, Peru, Mexico, Colombia, and Argentina (Table 2).

Although a considerable portion of the studies were conducted in partnership with non-Latin American institutions, the genotypic methods were executed outside Latin America only in small portion, highlighting the commitment of Latin American countries to advance *M. tuberculosis* research. Of the 345 articles, 20.9% carried out the genotypic methods through partnerships with 14 countries, mainly, US (12.5%), France (2.3%) and Spain (1.7%). The Latin American countries that performed the most genotypic methods through partnerships in approximately 70% of the collaborative studies, were Peru, Brazil, Colombia, and Mexico (Table 2). In almost half these studies (45.8%) the collaborations enabled WGS and were generally established with the US (66.7%).

The small number of studies carried out with partnerships between Latin American institutions highlighted the importance of strengthening and expanding the collaborative networks of TB research groups, particularly for countries with low scientific productivity and high TB burdens, such as Bolivia, El Salvador, Haiti, Panama, and the Dominican Republic. The increased frequency of collaborations established between the US and Latin American countries was likely driven by the US's significantly large investment in health and TB research and development [26,27].

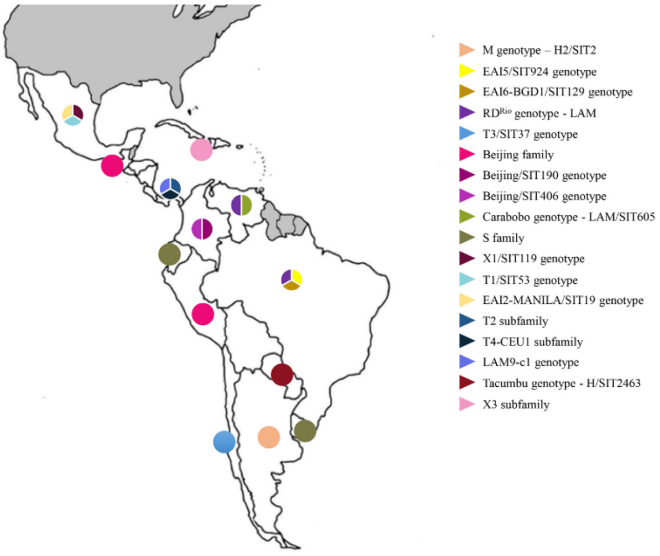
Main contributions of genotypic methods to Mycobacterium tuberculosis research in Latin America

M. tuberculosis has a long evolutionary history within Latin American population, but there is still no consensus as to when this interaction began. Pre-Columbian paleontological evidence suggests that the presence of *M. tuberculosis* in the Americas predates the contact of native people with European explorers [28–30]. In contrast, the Euro-American *M. tuberculosis* lineage predominance in Latin America suggests that *M. tuberculosis* was introduced and disseminated in Latin America during the European colonization between the 16th and 19th centuries [31].

As expected, the Euro-American lineage was predominant in Latin America, especially the Latin-American-Mediterranean (LAM), Haarlem and T families. Nonetheless, there were differences in the phylogeographic distribution of *M. tuberculosis* between Latin American countries and inside of each country. LAM (41.1%) was the predominant family in most Latin America countries. The Haarlem (20.4%) and T (18.7%) families, on the other hand, were intermittently the second and third most prevalent families. However, Haarlem was the most prevalent family in Bolivia and Peru, and T was significantly more prevalent in Mexico (data not shown).

The most striking differences in Latin American countries are presented in Figure 4 and described below. These differences probably resulted from the diverse colonization processes and migratory flows that have occurred over the centuries (Figure 5) [31–34].

Figure 4. Specific genotypes associated with Latin American countries.



Genotypes associated with Euro-American lineage in Latin America

Latin-American-Mediterranean family: Brazil, Venezuela, and Panama

In Brazil, the joint use of spoligotyping with IS*6110*-RFLP and MIRU-VNTR demonstrated the long evolutionary history of the RDRio genotype, described in Rio de Janeiro, and confirmed that it belongs to the LAM family, with a possible ancestor in the LAM9 subfamily [35–37]. It still showed specificities within the LAM subfamilies, with LAM1 and LAM2 representing only the RDRio genotype, LAM3 representing only wild-type genotypes, and LAM4-6 and LAM9 presenting both genotypes [35].

The RDRio genotype is remarkably frequent in Brazil, although its prevalence varies widely between regions (15.5% to 52%) [38,39]. In Venezuela it was reported in almost 70% of LAM strains [40]. However, it appears to have a limited contribution to the TB burden in Argentina and Paraguay, even though they share a border with Brazil. This limited contribution can be explained by the high proportion of the LAM3 subfamily in Argentina and by the low proportion of LAM1 and LAM2 in Paraguay [32,41].

In Venezuela, a considerable number of genotypes with regional specificities have been documented. An instance of this is the genotype SIT605, found predominantly in the state of Carabobo and in only two isolates outside the country [42,43]. Analyses of 45 SNPs showed that it belongs to the LAM family, and due to its geographical distribution, it was named "Carabobo" [42]. Méndez *et al*. [40] inferred, by using spoligotyping and 24-*loci* MIRU-VNTR, that the SIT605 genotype could belong to the LAM1 subfamily, and evidenced that none of the SIT605 strains harbored the RDRio genotype.

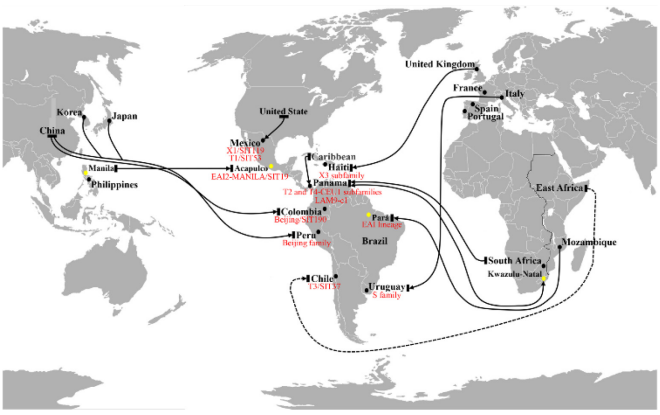
The use of the set of 24-*loci* revealed that all strains of the Carabobo genotype, regardless of region, had the same genotype profile for at least 21 *loci*, highlighting their high genetic similarity. The 24-*loci* MIRU-VNTR method confirmed the designation of the Carabobo genotype as belonging to the LAM family. Additionally this method demonstrated that the strains harboring the SIT1698, specific to Venezuela, seem to belong to the Carabobo genotype, since they shared the same genotype profile [40,42,44].

To elucidate the phylogenetic relationships among multidrug-resistant (MDR) strains in Panama, SNP-based analyses were performed. These analyses showed the presence of a large cluster belonging to the LAM9 subfamily (LAM9-c1 cluster) and composed of MDR strains. All strains in the LAM9-c1 cluster carried a specific combination of mutations in *katG*, *rpoB* and *rrs*, which conferred resistance to isoniazid, rifampicin, and streptomycin, respectively. The presence of this particular combination of mutations in all strains suggested continuous transmission from an already resistant strain, rather than independent acquisition of resistance [45].

Phylogenetic analyses also revealed a relationship between LAM9-c1 and the MDR/extensively drug-resistant (XDR) strain from Kwazulu-Natal (KZN), South Africa. These strains share SNPs absent in other LAM strains but have unique groups of SNPs that differ from each other. Therefore, these strains did not evolve from each other, but probably shared a close common ancestor that was able to tolerate the accumulation of a large number of mutations [45].

The data obtained inferred transmission of the ancestor between Panama and South Africa, but did not allow the direction of this transmission to be established. However, Panama had experienced two large African immigrations, which may have introduced the ancestral genotype of LAM9-c1 into the country [45,46]. Phylogeographic analyses based on complete genomes support that the LAM9-c1 and KZN genotypes were closely related but inferred independent introductions of the ancestor of these genotypes to Panama and South Africa, brought over by Europeans during the late 19th century [31].

Figure 5. Probable introduction routes of different genotypes in Latin America.



Continuous line: Introduction route established; Dashed line: Introduction route not established. Caribbean: Antigua, Barbuda, Aruba, Curaçao, Bahamas, Bermudas, Cayman Islands, Cuba, Dominica, Grenadines Islands, Guyana, Guadeloupe, Grenada, Jamaica, Martinique, Montserrat, Puerto Rico, Saint Lucia, Saint Barthelemy, Saint Kitts and Nevis, Dominican Republic, Haiti, Island of Saint Martin, Saint Vincent, Suriname, Trinidad, Tobago, Turks and Caicos Islands and Virgin Islands.

Haarlem family: Argentina and Paraguay

During the 1990s, it was possible to record the occurrence of nosocomial outbreaks caused by *M. tuberculosis* MDR strains in Argentina using the IS*6110*-RFLP method. Although the initial outbreaks involved patients living with HIV [47,48], MDR strains spread rapidly among immunocompetent people, with reports of secondary micro-epidemics. The second and largest MDR-TB outbreak occurred in the reference hospital for infectious diseases in Buenos Aires. The strain called "M" was responsible for 92% of the cases. Two main variants of this strain, "Mm" and "Mn" were differentiated by the presence of an additional copy of IS*6110* in the Mn variant (9 copies). It was notable that the M strain, which was associated with resistance to at least five drugs and rapid disease progression, was responsible for several secondary outbreaks [48–52].

WGS analysis enabled the reconstruction of the evolutionary trajectory of the M strain during the outbreak, suggesting that its ancestor had already acquired resistance to isoniazid, rifampicin, and streptomycin in the early 1970s. Estimates suggested that the ancestor of the M strain could already be defined as pre-XDR by 1979 due to the acquisition of resistance to three more drugs, ethambutol, pyrazinamide, and kanamycin [53]. The phylogenetic analysis based on SNPs identified the M strain as Haarlem due to the presence of the three SNPs characteristic of this family in codons 754, 172 and 65, and this was consistent with the spoligotyping data (WGS: sub-lineage 4.1.2.1; spoligotyping: subfamily H2/SIT2) [52–54]. In addition, based on IS*6110*-RFLP and WGS data and the increasing incidence of MDR-TB, it was suggested that few M strains were circulating in Argentina before entering the hospital, where it disseminated among HIV-positive patients and dispersed to other sites [53].

In Paraguay, the combined use of IS*6110*-RFLP, MIRU-VNTR, and spoligotyping allowed the identification of genetic patterns restricted to the country, which may have evolved from strains introduced during colonization and subsequently prospered locally. This scenario could be exemplified by the genetic pattern identified in a prison in Asunción, was named “Tacumbu”, in honor of the prison, and classified by spoligotyping as belonging to the Haarlem/SIT2643 family [55].

The fact that the M and Tacumbu genotypes are not found in people outside of Argentina and Paraguay reinforced the endemicity of these genotypes to these populations [52,55].

T family: Panama, Chile, and Mexico

The T family was the most representative in Brazil according to the SITVIT2 database. However, the frequency of this family was significantly higher than LAM and Haarlem only in Mexico. The T1 subfamily, especially the T1/SIT53 genotype, was the most common among the regions studied. It should be noted that the predominance of T1/SIT53 was a result of centuries of extensive contact with the US, and that this genotype was found throughout the Mexican territory [56–58].

Although the T1/SIT53 genotype was frequently described, T2, T4-CEU1 and T3/SIT37 were rarely found in Latin American countries in proportions above 2%. Nonetheless, T2 and T4-CEU1 genotype were highly represented in Panama and the T3/SIT37 genotype in Chile. T3/SIT37 was usually found in East Africa, however it seemed to have a high capacity for adaptation and dissemination in Chile even though migration from Africa to Chile was rare. In contrast, the high prevalence of the T2 and T4-CEU1 subfamilies in Panama could be explained by the migration of Caribbeans and South Africans, respectively, during the construction of the Panama Canal in the early 20th century [46,59].

Other families: Haiti, Ecuador, and Uruguay

In insular Central America, there was great genetic diversity of *M. tuberculosis* due to the movement of African slaves, Spanish, French, and later the English, which may explain the significant presence of the X family in Haiti [60,61]. The X3 subfamily was infrequent in most countries, not exceeding 2% of the genotypes of *M. tuberculosis* according to the SITVIT2 database. However, in Haiti the X3 subfamily was highly disseminated. This subfamily was found mainly in the US and in the United Kingdom, which had strong influences in the Caribbean over the years. Therefore, the X3 subfamily was probably introduced in Haiti during the Anglo-Saxon colonization process [61, 62].

The genetic diversity of *M. tuberculosis* in Uruguay remained poorly understood, and what was known about the genotype prevalence referred to isoniazid-resistant strains. The presence of the S family was noteworthy, and, unlike the rest of Latin America, it had strongly contributed to the resistant TB case load. This prevalence could be partially explained by the great migration of Italian communities to Uruguay in the 20th century, which continued to represent a considerable portion of the Uruguayan population [63]. More importantly, the highest proportion of the S family in South America to date was found in Ecuador (13.1%),

since the prevalence of this family in Uruguay refers to resistant strains [24].

Genotypes associated with other lineages in Latin America

East-Asian lineage - Beijing family: Peru, Colombia, and Guatemala

This family was phylogenetically divided into two main subfamilies, termed modern and ancient, considering the evolution time between these subfamilies and the ancestral genotype. The modern subfamily is predominant in China, although it is typically found throughout the world, whereas the ancient subfamily is predominant in Japan and Korea [21,64]. The Beijing family was found in low proportions in Latin America, but its prevalence had been increasing in Peru (16.4%) and Colombia (15.6%) [65,66].

Peru was home to the modern and ancient subfamilies due to the migratory flows that occurred in the late 19th century from China, Japan, and later, from Korea in the late 19th century. Peru is still considered the main source of transmission of the Beijing family to other Latin American countries [64,65]. The use of MIRU-VNTR allowed analysis of genetic variations among Beijing strains in Peru, which were not detected by spoligotyping. High rates of active and continuous Beijing transmission were identified, mainly with strains harboring the PCT001 genotype. This methodology also revealed that most XDR Beijing strains in Peru were phylogenetically closer to Japanese strains considering their similarity to the common Japanese MIRU pattern, MIRU International Type 11 (MIT; code assigned by the SITVIT2 database) [64,67].

As in Peru, in Colombia, there was also a high prevalence of Beijing strains. However, the Beijing family was only associated with highly resistant phenotypes in Colombia, due to the SIT190 genotype, which is predominant in the US and China, but rare in Latin America [34,66,68]. SIT190 was responsible for almost all the Beijing cases in Buenaventura, Colombia, and was strongly associated with MDR-TB and new cases, indicating active transmission of this resistant genotype. The city of Buenaventura is one of the largest cities in Colombia, with strong tourist and commercial activities and is home to the country's main port, a place that appeared to have been the gateway for the Beijing family through Asian immigration [34,66].

The use of MIRU-VNTR in Colombia enabled more robust analyses of Beijing, to understand the distribution of SIT190 genotype within the country. Although highly homogeneous, these Beijing strains were subdivided into 12 distinct genotypic patterns, comprising 4 orphan strains and 8 phylogenetically connected patterns. However, some genotypes appeared to be more distant and had greater variability at the analyzed *loci*. This suggested the emergence of virulent clones, strongly associated with drug resistance [34,68]. In addition, other rare genotypes of the Beijing family in Latin America had been described in Colombia, among them SIT406. However, it was important to note that SIT406 had only been documented in about 20 strains worldwide [69,70].

Altogether, the data from Peru and Colombia supported the hypothesis of multiple introductions of the Beijing family in Latin America [34,64–67,70].

Finally, in Guatemala WGS analyses were performed to investigate the phylogenetic history of *M. tuberculosis*. Saelens *et al*. observed the presence of emerging fluoroquinolone-resistant Beijing strains, predominantly from the modern subfamily, which differed from each other by up to 31 SNPs, indicating the presence of a recently shared parent strain. These strains were also involved in a community outbreak initiated in a prison, where two closely related isolates were obtained from inmates at different times. Since the strains diverged in up to 31 SNPs, the outbreak had probably been occurring for several years, allowing the evolution of these strains within the prison [71].

East-African-Indian lineage: Brazil and Mexico

Strong phylogeographic specificities were observed in different regions of Brazil resulting from different migratory processes that occurred in each region [33,72–75]. We highlight the significant presence of the East-African-Indian (EAI) lineage in the northern region, specifically in Pará, in almost 10% of the TB cases. This was the result from the long history of forced migration of African slaves from East Africa, mainly from Mozambique, between the 16th and 19th centuries, as evident from the correlation between the strains identified in both countries using 24-*loci* MIRU-VNTR and spoligotyping [33,74,75]. The genotypes EAI5/SIT924 and EAI6-BGD1/SIT129 were related to recent and specific genotypes in Pará, indicating the ability of the EAI lineage to spread in this population [33,74].

The EAI lineage was present in Acapulco, Mexico, where there was a high clustering of EAI2-Manila family isolates (26.2%). We highlight the EAI2-Manila/SIT19 genotype, which seemed to behave as an emerging genotype due to the high transmission rate. The high prevalence of the EAI2-Manila family may be explained by the long history of commercial relations

with the Philippines between 1565 and 1815, a period when both countries were Spanish colonies [76–78].

Conclusions

The present study highlights and dissects the implementation of *M. tuberculosis* genotyping methods in Latin America. The review of the literature showed that spoligotyping was the most widely used method in Latin America but since 2014, its use had been decreasing in parallel with an increase in the use of MIRU-VNTR and WGS. In addition, it shows that a considerable part of the studies was conducted in partnership with Latin American and/or non-Latin America institutions. However, only a small proportion of studies needed partnerships to perform the genotypic methods, reflecting the commitment of Latin American countries to the advancement of TB research and development. We highlight that Brazil, Mexico, Argentina, Peru, and Colombia contributed almost 90% of the Latin American articles and a predominance of the Euro-American strain in Latin America was observed.

Extensive use of genotypic methods improved our understanding of the population structure of *M. tuberculosis* by revealing genetic factors that may influence transmission, *M. tuberculosis* transmission hotspots, and populations infected with highly virulent genotypes. Moreover, the development of a database of *M. tuberculosis* strains, as presented on the Community of Portuguese-Speaking Countries-TB website [79], would be interesting for Latin America, considering the ease of interaction among Latin peoples and their dispersion to all continents. Strategies such as these would allow greater articulation between research and public health services, ensuring that the genotypic methods evaluated can reflect on better therapeutic strategies and the development of adequate TB control protocols.

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Authors' contribution

YCIN: acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be submitted; AJR: analysis and interpretation of data, drafting the article, final approval of the version to be submitted; NXM: acquisition of data, final approval of the version to be submitted; JV: analysis and interpretation of data, final approval of the version to be submitted; JP: analysis and interpretation of data, final approval of the version to be submitted; IBR: analysis and interpretation of data, final approval of the version to be submitted; PEAS: analysis and interpretation of data, final approval of the version to be submitted; AVG: conception and design of the study, acquisition of data, analysis, and interpretation of data, drafting the article, final approval of the version to be submitted.

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