Genetic diversity of *Mycobacterium tuberculosis* in Ecuador assessed by MIRU-VNTR analysis of sputum smear samples from primary health care centres

Patricia Jiménez-Arias[1,2,3], Stalin Bedón[2,4], Bryan Cevallos-Rocha[1], Alexis Naula-Guambo[1], Héctor Javier Sánchez-Pérez[3,5], Natalia Romero-Sandoval[2,3]

[1]Departamento de Ciencias de la Vida y de la Agricultura. Universidad de las Fuerzas Armadas – ESPE. Sangolquí. Pichincha. Ecuador. [2]School of Medicine. Universidad Internacional del Ecuador. Quito. Ecuador. [3]GRAAL. Grups de Recerca d'Amèrica i Àfrica Llatines. Barcelona. España. [4]Ministry of Public Health of Ecuador. Quito. Ecuador. [5]El Colegio de la Frontera Sur (ECOSUR). El Colegio de la Frontera Sur Health Department. San Cristóbal de Las Casas. Chiapas. México.

Summary

Background: Tuberculosis is caused by *Mycobacterium tuberculosis* (MTB), with 10.8 million cases and 1.25 million deaths in 2023. Understanding molecular epidemiology and improving diagnosis requires MIRU-VNTR genotyping. This study aimed to determine the genetic diversity of MTB in Quito, Ecuador, using DNA extracted from Ziehl–Neelsen-stained smear slides (ZN-s) collected between 2019 and 2021 in primary care centres.

Material and methods: A cross-sectional-experimental design was performed with 11962 ZN-s, of which 5852 met the inclusion criteria. All smear-positives (n = 37) and a random sputum of smear-negatives (n = 203) were analysed. The variables examined were gender, age and blinded rechecking of ZN-s. Genetic diversity was explored by 12-MIRU-VNTR.

Results: Of 5852 ZN-s, 0.6% were positive. Read and re-read concordance was high (Kappa = 0.99). Young men were more likely to have tuberculosis ($p < 0.05$). MIRU-types were obtained from 9/37 positive ZN-s: 66.7% sublineage EAI-lineage 1, 22.2% sublineage S-lineage 4, and 11.1% unidentified.

Conclusions: This first Ecuadorian study using 12 MIRU-VNTR on stored sputum smears suggests Indo-Oceanic lineage 1 is the most frequent followed by Euro-American Lineage 4. A larger sample is needed to confirm lineage prevalence and fully assess the genotyping approach directly from stored positive ZN-stained smears.

Key words:
Mycobacterium tuberculosis.
Genotyping. Sputum smear.
Primary care. Ecuador.

Diversidad genética de *Mycobacterium tuberculosis* en Ecuador evaluada mediante análisis MIRU-VNTR de muestras de esputo obtenidas en atención primaria

Resumen

Fundamentos: En 2023, *Mycobacterium tuberculosis* (MTB) provocó 10.8 millones de enfermos y 1.25 millones de muertes. La genotipificación MIRU-VNTR es necesaria para mejorar el diagnóstico y comprender la epidemiología molecular. Determinamos la diversidad genética de MTB utilizando ADN extraído de muestras de frotis teñidas con Ziehl-Neelsen (ZN-s) recolectadas en centros de atención primaria (CAP) en Quito, Ecuador entre 2019 y 2021.

Material y métodos: Se incluyeron 5852/11962 ZN-s en un estudio transversal y experimental. Se analizaron todos los frotis positivos (n = 37) y una muestra aleatoria de esputos negativos (n = 203). Se analizó sexo, edad y relectura de ZN-s, a doble ciego. Se exploró la diversidad genética mediante 12 loci MIRU-VNTR.

Resultados: 0,6% ZN-s resultaron positivas. La concordancia entre lectura-relectura fue alta (Kappa = 0,99). Los hombres jóvenes presentaron mayor probabilidad de tener tuberculosis ($p < 0{,}05$). Se obtuvieron MIRU-tipos en 9/37 ZN-s: 66,7% del sublinaje EAI-linaje 1; 22,2% del sublinaje S-linaje 4; y, 11,1% no fueron identificados.

Conclusiones: Este primer estudio ecuatoriano que utiliza 12 MIRU-VNTR en frotis de esputo almacenados sugiere que el linaje indo-oceánico 1 es el más frecuente seguido por el euro-americano linaje 4. Se necesita una muestra más amplia para confirmar la prevalencia del linaje y evaluar completamente el enfoque de genotipado directamente a partir de frotis positivos teñidos con ZN almacenados.

Palabras clave:
Mycobacterium tuberculosis.
Genotipificación. Frotis de esputo.
Atención primaria. Ecuador.

Correspondencia: Natalia Romero-Sandoval
E-mail: nromero@uide.edu.ec

Introduction

Mycobacterium tuberculosis (MTB) is a facultative intracellular microorganism with unique phenotypic attributes that give it resistance to several antibiotics, along with the ability to survive in harsh environments. This bacterium is responsible for tuberculosis (TB), an infectious disease that primarily affects the lungs and is transmitted when a sick person coughs, speaks or sputters, is treatable and the means exist to prevent deaths from this cause. In 2022, 10.6 million people contracted TB and about 1.30 million died from it globally[1]. In that year, the Ecuadorian National Tuberculosis Prevention and Control Strategy (ENPCTB) of the Ministry of Public Health (MoPH) reported 6 872 TB cases, a cumulative incidence rate of 38 cases per 100 000 population[2].

In Ecuador, diagnosis is generally based on sputum smear, which is performed in some Primary Health Care Centres (PHCC) that have the necessary laboratory facilities. In at risk groups, culture and nucleic acid amplification tests are also used to detect both MTB complex bacteria and rifampicin resistance[3].

Molecular epidemiology of TB, on the other hand, allows characterisation of active transmission, distinguishing between recent infection and reactivation, determining whether all strains have similar epidemiological characteristics in populations, understanding transmission dynamics at the population or cluster level, and identifying outbreak[4].

MIRU-VNTR genotyping is a method based on Polymerase Chain Reaction (PCR) amplification of 12, 15 or 24 loci that calculates their size and the number of repetitive units[5,6]. Amplification of 12 MIRU-VNTR loci has moderate discriminatory ability to determine lineages and sub lineages of MTB isolates from mycobacterial cultures[7]. The identification of loci with higher discriminatory power allows the selection of specific loci that can save time and costs and ensure reliable genotyping results[8]. This scheme was selected as it offers a balance between discriminatory power and operational feasibility in resource-limited settings, in situations where culture samples are unavailable or difficult to obtain, or when rapid typing is required, such as for outbreak control[6,7]. In this context, this study aimed to determine the genetic diversity of MTB using the MIRU-VNTR technique with DNA recovered from sputum smear collected from three PHCC units in Quito, Ecuador, 2019-2021, and as secondaries objectives, it sought to analyse the association between the results of the sputum smears analysed with age and sex, and to conduct an analysis of the concordance between the initial reading and the rereading of the sputum smears. Despite being recognised for its effective implementation of the ENPCTB, Ecuador's capital (Quito) has not been the focus of epidemiological studies evaluating novel molecular techniques, such as the use of DNA recovered from sputum smears for strain typing, drug resistance profiling, and transmission tracking. This gap is particularly notable given the city's role as a national reference for TB control efforts.

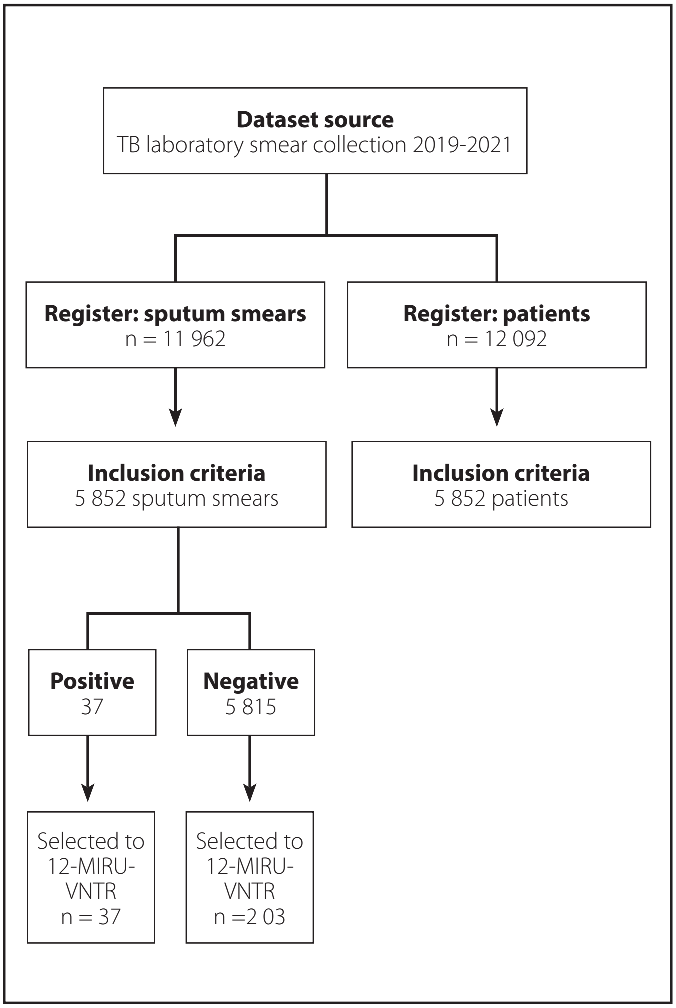
Materials and methods

The present study had two components: the first, a cross-sectional design, and the second, an experimental study. The STROBE guidelines for observational studies were used to structure this report[9]. The sputum smear analysed in this study correspond to the period 2019-2021 and come from the TB laboratory smear collection of all (three) PHCC of MoPH, located in the south-eastern area of Quito, Ecuador, which kept Ziehl-Neelsen stained (ZN-s), coded smears and an electronic data-base standardised by the ENPCTB. These three PHCC provide the TB laboratory diagnostic test for TB in the aforementioned area, which has an estimated population of approximately 90,000 inhabitants, of which around 70% are covered by MoPH[10]. Both positive and negative smears were considered, one per person with respiratory symptoms (PRS) over 18 years of age who had not received TB treatment and who had the required demographic information. Sputum smears that were outside the study period, in poor staining condition, broken or with illegible codes were excluded, as well as those corresponding to treatment controls, to persons under 18 years of age and who did not have complete information required in the database.

Demographic variables analysed were gender and age. The accuracy and reproducibility of smear microscopy readings were assessed by comparing results from the health unit laboratory with re-readings in the research laboratory, categorizing results by AFB count and as positive/negative. The variables DNA purity A260/A280, A260/A230 and DNA concentration were handled as continuous variables.

A new code was assigned to all sputum smears considered in the study to mitigate possible selection and reading biases. The only source of information used to obtain the PRS data was the standardised electronic database of the ENPCTB of the PHCC studied. The smears were placed in an airtight container made of resistant plastic to protect them from shocks and vibrations during transport. Bubble wrap was used for packaging to avoid gaps between the smears. The containers were identified with numerical codes. The sputum smears were kept at room temperature. The transport service between the health units and the research laboratory ensured a quick and direct arrival to avoid delays that could affect the samples, accompanied by the shipping form.

Figure 1. Sample flowchart summarizing sample inclusion.



To minimise confounding, all variables available in the electronic database were considered, and both positive and negative smears for re-reading followed by DNA identification. Figure 1 illustrates the sample flowchart summarizing sample inclusion.

Extraction of DNA from smear microscopy slides after storage

The sputum smears were pre-treated with Neo-Clean (Sigma-Aldrich^{TM}) and Net-10 buffer (Laboratory-prepared samples) for five minutes and washed with autoclaved distilled H_2O. The sputum smear was collected in a 1.5 mL microtube with 200 uL of TE buffer (Tris - EDTA). The Minikit QIAamp DNA™ protocol for isolation of genomic DNA from Gram-positive bacteria was used according to the manufacturer's instructions. Finally, the extracted DNA was quantified by spectrophotometry (Multiskan Sky Microplate Spectrophotometer™), determining the concentration (ng/uL) of DNA and the A260/230 and A260/280 ratios[11].

Genotyping (MIRU-VNTR)

Amplification of 12 molecular markers (MIRU 02, 04, 40, 10, 16, 20, 20, 23, 24, 26, 27, 31 and 39) was performed as described in Supply, *et al.,* 2006[6]. DNA at 5 ng/uL of H37Rv grown in Löwenstein-Jensen (LJ) medium was used as a positive control and ultrapure H_2O as a negative control. For PCR amplification, the enzyme Platinum™ Taq DNA Polymerase from Thermo Scientific™ was used in a final volume of 10 uL with 3 uL of total DNA extracted from the samples. To visualise the amplification of the PCR products, horizontal gel electrophoresis was performed in 1.8% agarose. Thermo Scientific™ 100 bp GeneRuler DNA was used as molecular weight marker and the electrophoretic run was performed at 100 V, 300 mA for 100 minutes.

MIRU genotyping

To obtain the molecular weight and the number of repeats of each molecular marker, Image Lab software version 6.1 was used, the mirutype was analysed on the free access platform www.miru-vntrplus.org to determine the lineage and sublineage belonging to each sample accompanied by its corresponding dendrogram.

Data analysis

Data were entered into Excel and exported to SPSS v29 for further analysis. The variables are described in terms of relative and absolute frequencies. Associations between smear result and factors under study were assessed using binary logistic regression. The results are expressed in terms of crude and adjusted OR and their 95% CI. Cohen's Kappa coefficient was used to assess concordance between smear reading and re-reading results to verify that routine health unit readings matched the research team's re-reading, ensuring correct classification of samples for DNA extraction. Kruskal-Wallis Test was applied to compare medians between three or more independent groups. A *p*-value for a two-tailed test of <0.05 was considered statistically significant.

Ethical considerations

This study was approved by the Human Research Ethics Committee of San Francisco General Hospital (CEISH-HGSF), code CEISH-HGSF-2022-30.

Results

The database contained 12 092 registers corresponding to PRS, both diagnostic and control, from which 11 962 smears were obtained. Among the 5 852 (48.9%) sputum smears that met the inclusion criteria, all positive smears (37/5852; 0.6%) were analysed. Of those negative (5815/5852), 203 (3.5%) were selected at simple random (size chosen for convenience due to time, logistical and budgetary constraints), so that the total sample analysed were 240 sputum smears.

Table 1 shows demographic characteristics of Ziehl–Neelsen-stained smear slides from PRS. Of the 37 positive smears, a positivity rate according to sex was 0.9% and 0.4%, for men and women, respectively (Chi-square = 5.1, *p* <0.01, prevalence ratio = 2.1; 95% CI 1.1 - 4.0). In multivariate analysis, sputum smear positivity against sex and age was correctly classified in 99.4% of cases. There was enough evidence to suggest association between males (adjusted OR 2.15; 95% CI 1.11 - 4.15) and the probability of having a positive smear test at younger age (adjusted OR 0.98; 95% CI 0.96 - 0.99).

Table 1. Demographic characteristics of Ziehl–Neelsen-stained smear slides from persons with respiratory symptoms from three primary care centres, Quito, Ecuador, 2019-2021.

	Cases (smear +)	Non cases (smear -)	Prevalence (CI 95%)
All	37	5 815	0.63% (0.48-0.83)
Sex-females	15	3 426	0.44% (0.64-1.29)
Sex-males	22	2 389	0.91% (0.29-0.66)
Age-years, mean (CI 95%)	39.2 (33.6-44.9)	45.2 (44.7-45.7)	

Analysis of concordance between diagnostic reading and re-reading of sputum smears

Of the 240 smears analysed, 99.5% of the negatives in the re-reading corresponded to negatives in the initial reading, while 100% of the positives corresponded to positives (Cohen's Kappa coefficient = 0.98; 95% CI 0.97-1.00; *p* <0.01).

DNA extraction from smear microscopy slides after storage

DNA extraction analysis was performed on the 37 positive smears: 6 with less than 10 AFB per field, 18 with one cross (+), 5 with two (++) and 8 with three (+++). A mean genomic DNA concentration was obtained by Minikit QIAamp^{TM} of 21.7 ng/uL, (SD 37.1), median = 8.1 ng/uL, Q1 = 4.7 ng/uL and Q3 = 27.7 ng/uL. For the absorbance ratio A260/280 a mean of 1.57 (SD 1.18), median =1.38, Q1 = 1.15 and Q3 =1.70 was obtained. For the absorbance ratio A260/230, the following values were obtained: mean = 0.98 (SD 0.91), median = 0.67, Q1 = 0.27 and Q3 = 1.39. In terms of 260/280 purity, 27/37 (72.9%) had presence of aromatics, proteins or phenols and 10/37 (27.1%) showed optimal conditions (Table 2). Table 3 shows the values obtained and the comparison between groups. When comparing the medians of genomic DNA concentration and absorbance ratio according to the smear test categories, no significant differences were observed between groups (*p* = 0.45).

Table 2. Summary of data obtained from Ziehl–Neelsen-stained smear slides (ZN-s), DNA extraction and genotyping by MIRU-VNTR.

No.	DNA concentration (ng/uL)	A260/280	A260/230	ZN-s reading	ZN-s re-reading	MIRU 2	MIRU 4	MIRU 40	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39
1	9.59	1.21	0.16	"++"	"+"	-	-	-	-	-	-	-	-	-	-	3	-
2	5.20	1.65	0.22	"+++"	"+++"	2	-	-	-	-	-	-	2	-	-	-	-
3	1.23	1.12	0.92	"+"	"5 AFB"	-	-	-	0	-	-	-	-	-	-	0	-
4	84.29	1.63	1.70	"+++"	"+++"	-	-	-	-	-	5	-	-	-	-	0	-
5	32.65	1.30	1.79	"+++"	"+++"	-	-	-	-	-	-	-	-	-	-	0	-
6	5.51	1.42	0.51	"+++"	"+++"	-	-	4	-	-	-	-	-	-	-	3	-
7	32.65	1.89	1.29	"++"	"+"	3	3	3	4	4	3	5	2	8	5	4	3
8	5.92	1.38	2.46	"+++"	"+++"	3	-	1	-	-	-	5	-	-	4	0	-
9	32.24	1.85	1.08	"+++"	"+++"	-	-	-	-	-	-	-	-	-	-	0	-
10	13.57	1.99	0.38	"+"	"+"	-	-	-	-	-	-	10	-	5	-	-	5
11	8.88	1.36	0.70	"+++"	"+"	-	-	-	0	-	-	-	-	-	-	0	0
12	63.88	1.87	2.37	"+++"	"+++"	3	-	2	-	-	-	7	-	-	-	0	-
13	3.27	1.33	0.37	"+"	"+"	-	-	4	6	-	-	-	-	-	5	4	4

(continue)

Table 2. Summary of data obtained from Ziehl–Neelsen-stained smear slides (ZN-s), DNA extraction and genotyping by MIRU-VNTR *(continuation).*

No.	DNA concentration (ng/uL)	A260/280	A260/230	ZN-s reading	ZN-s re-reading	MIRU 2	MIRU 4	MIRU 40	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39
14	3.47	1.10	0.77	"++"	"+"	3	2	-	-	-	0	-	-	-	-	-	0
15	24.18	1.60	0.59	"+++"	"++"	-	-	-	-	-	-	-	-	-	-	7	0
16	15.31	0.97	0.74	"+++"	"++"	-	-	-	0	-	-	-	-	-	-	0	0
17	8.16	1.25	3.20	"++"	"+"	3	-	2	-	-	2	6	2	-	5	4	0
18	1.22	0.57	0.22	"++"	"+"	-	-	-	0	-	-	-	-	-	-	0	0
19	11.22	1.25	1.31	"+++"	"+++"	1	3				-	-	-	-	-	-	8
20	5.41	1.77	0.22	"+"	"5 AFB"	-	-	-	6	-	3	6	-	7	-	-	-
21	50.51	1.24	0.60	"+"	"+"	-	-	-	0	-	-	-	-	-	-	-	-
22	4.39	1.08	0.54	"+"	"5 AFB"	-	-	-	7	-	-	-	-	3	-	-	-
23	6.63	1.86	0.25	"++"	"++"	3	3	4	7	6	3	5	2	5	5	4	3
24	12.14	1.57	3.31	"+"	"1 AFB"	-	-	-	-	-	-	-	-	-	-	-	-
25	5.71	1.87	0.29	"+++"	"+"	-	-	-	-	-	-	-	-	-	-	-	-
26	7.86	0.91	0.27	"++"	"++"	1	-	-	3	-	2	7	-	6	5	-	-
27	11.02	1.54	0.68	"++"	"+"	-	-	-	-	-	-	-	-	-	-	-	-
28	5.92	2.9	1.07	"+++"	"+"	-	-	-	-	-	-	-	-	-	-	0	-
29	13.16	1.19	3.15	"+"	"8 AFB"	-	-	-	-	-	-	-	-	-	-	-	-
30	0.51	0.00	0.11	"+"	"+"	-	-	-	-	-	-	-	-	-	-	4	-
31	5.29	1.23	0.41	"++"	"+"	-	-	-	-	-	-	-	-	-	-	-	-
32	0.11	1.04	0.26	"++"	"+"	-	-	-	-	-	-	-	-	-	-	-	-
33	31.22	1.40	0.68	"+"	"+"	-	-	-	-	-	-	-	-	-	-	-	-
34	205.60	1.43	1.47	"+"	"+"	3	-	-	-	-	-	7	2	-	4	4	3
35	69.29	1.53	1.92	"+"	"4 AFB"	3	3	3	4	1	2	6	3	7	4	5	3
36	2.45	8.00	0.00	"+"	"+"	-	-	-	-	-	-	6	2	-	-	0	-
37	3.98	1.03	0.43	"+++"	"++"	3	2	4	5	-	3	6	2	6	5	4	3

AFB: Acid-Fast Bacilli; Positive (+): 10–99 AFB observed in 100 fields; Positive (++): 1–10 AFB per field in 50 fields; Positive (+++): More than 10 AFB per field in 20 fields[43].

Table 3. Determination of DNA concentration and purity in positive Ziehl–Neelsen-stained smear slides analysed from three primary care units Quito, Ecuador, 2019-2021.

Results	Number of sputum smears (%)	DNA concentration (ng/ml)		Purity A260/280		Purity A260/230	
		Median	IQR	Median	IQR	Median	IQR
< 10 AFB	6 (16.2)	8.77	8.26	1.36	0.423	1.42	2.208
1 (+)	18 (48.7)	7.03	9.61	1.34	0.573	0.50	0.485
2 (++)	5 (13.5)	7.85	8.68	1.03	0.630	0.43	0.320
3 (+++)	8 (21.6)	21.73	34.64	1.52	0.340	1.5	0.998
Total	37 (100.0)	8.1	17.80	1.38	0.555	0.67	1.325

12-MIRU-VNTR genotyping

The 12 MIRU-VNTRs were obtained for 8.1% (3/37) sputum smears, 11 to 5 MIRU-VNTRs for 16.2% (6/37) and less than 5 MIRU-VNTRs for 75.7% (28/37) smears. Six of the 37 samples showed optimal purity conditions. For the bioinformatics analysis, only samples with data from 5 or more MIRU-VNTR loci, representing 24.3% (9/37) were considered. Figure 2 shows the dendrogram detailing the sublineage of the samples under study, 66.7% (6/9) belonging to sublineage EAI-lineage 1, 22.2% (2/9) to sublineage S-lineage 4, and 11.1% (1/9) could not be identified. In this analysis the positive control H37Rv was included for validation of the results. The most discriminatory MIRU-VNTR loci were found to be the 1.644 locus (MIRU 16) with an allelic diversity (AD) of 0.96, followed by the 2,996 locus (MIRU 26) with an AD of 0.91 and the 960 locus (MIRU 10) with an AD of 0.88, while the 154 locus (MIRU 02) was the least dis-criminatory with an AD of 0.43.

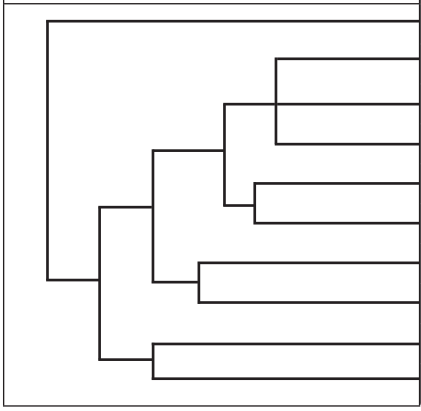
Discussion

This study analysed stored ZN-stained slides from three PHCC of south-eastern area of Quito, the largest cities in Ecuador, to determine the genetic diversity of MTB. The main challenge was precisely accessing ZN-s stored (2019-2021) at the PHCC for DNA extraction, as this would have been different if the slides had originated from a specialized TB laboratory. To our knowledge, this is the first study in Ecuador, although this technique has been known since 2003[12]. The use of the 12-loci MIRU-VNTR as a first-line method for MTB genotyping from sputum smears was considered, seeing that it is an internationally validated PCR-based technique, enables cluster screening in most scenarios, offers excellent portability, and is comparable through databases such as MIRU-VNTRplus. Given its lower cost and faster turnaround time, it is ideal for laboratories with limited resources or for continuous population-based surveillance. Nevertheless, the 12-loci set has lower discriminatory power than 15/24-loci panels, particularly for lineages such as Beijing; therefore, 15/24-loci or WGS panels will be considered for future studies if necessary. This strategy aligns guidelines and reference practices, balancing epidemiological validity, cost, turnaround time, and international comparability[13–16].

To provide clear context on the origin of the stored sputum samples, an initial epidemiological analysis was conducted. Men showed higher smear-positive PTB, consistent with community studies (Malaysia 2015-2019)[17]. The overall mean age of the PRS was 45.2 years. This pattern aligns with a 2001 study in Andean Ecuador, which reported the highest smear-positive PTB among men aged 35–44[18]. It is important to consider given the economic and occupational impact on this productive age, supporting with other studies[19]. The slides analysed were from PRS who sought consultation at health services in the studied area and do not represent the overall TB situation there; therefore, this fact needs to be considered when assessing the results.

In relation to the smear microscopy result, a low rate of TB positivity (0.6%) in PRS suspected of having TB was noted. Other study found rates of 1.7%[20]. To date, no studies from Ecuador have specifically examined this issue and this work represents an initial analysis of documented and stored ZN-s slides at the primary care level. The low sensitivity of Ziehl-Neelsen microscopy -below 10^4

Figure 2. UPGMA dendrogram based on 12-MIRU-VNTR from positive Ziehl–Neelsen-stained smear slides analysed from three primary care centres from Quito, Ecuador, 2019-2021.

Tree	ID	Species	Lineage	MIRU 02	MIRU 04	MIRU 40	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39
	Control +	*M. tuberculosis*$^{(u)}$	H37Rv$^{(s)}$	2	3	1	3	2	2	6	1	3	3	3	2
	7	*M. tuberculosis*$^{(s)}$	EAI$^{(m)}$	3	3	3	4	4	3	5	2	8	5	4	3
	23	*M. tuberculosis*$^{(s)}$	S$^{(s)}$	3	3	4	7	6	3	5	2	5	5	4	3
	34	*M. tuberculosis*$^{(s)}$	EAI$^{(s)}$	3	-	-	-	-	-	7	2	-	4	4	3
	17	*M. tuberculosis*$^{(s)}$	EAI$^{(s)}$	3	-	2	-	-	2	6	2	-	5	4	0
	37	*M. tuberculosis*$^{(s)}$	EAI$^{(s)}$	3	2	4	5	-	3	6	2	6	5	4	3
	13			-	-	4	6	-	-	-	-	-	5	4	4
	26	*M. tuberculosis*$^{(s)}$	EAI$^{(s)}$	1	-	-	3	-	2	7	-	6	5	-	-
	8	*M. tuberculosis*$^{(s)}$	S$^{(s)}$	3	-	1	-	-	-	5	-	-	4	0	-
	35	*M. tuberculosis*$^{(s)}$	EAI$^{(s)}$	3	3	3	4	1	2	6	3	7	4	5	3

bacilli/mL for diagnosing TB is well recognized[21]; however, in this study other reasons could explain the low positivity rate observed, such as the impact of the COVID-19 pandemic[22], early-stage or paucibacillary PTB, or limited laboratory experience[23]. The concordance analysis between reading and re-reading in our study was 99%, a result consistent with other studies on the subject (95% - 100% concordance between laboratory technicians)[24]. This ensured correct sample classification for DNA extraction.

The second step involved the experimental design, which showed that DNA extraction from stored sputum smears was consistent with the findings of Ruiz-Fuentes, *et al.*, 2015, who compared *M. leprae* DNA extraction methods and found that the Minikit QIAamp presented significantly different concentrations and the highest purity (1.65 ± 0.17 ng/µL, 1.44 ± 70.09 of 260/280) in smears with the highest number of AFB[11]. This result was also consistent with those of Van Der Zanden, *et al*., 2003; Carcelén, *et al.*, 2017; Tarhan, *et al.,* 2009 who evaluated molecular techniques for detecting MTB and identifying its lineages, finding that the QIAamp Minikit consistently provided superior DNA yield and purity compared to other extraction methods. In experiments aimed at enhancing lineage assignment and detecting rifampin resistance directly from Ziehl–Neelsen–stained slides or sputum specimens, QIAamp produced much higher DNA quantities and optimal 260/280 purity ratios, particularly in samples with a high bacilli count[12,25,26]. This method has demonstrated efficiency in obtaining high quality DNA, even in degraded samples[27,28].

Genotyping by amplification of 12 loci has been used to study MTB strains in Brazil[7], with results comparable to RFLP-IS6110, demonstrating similar discriminatory power and concordance. DNA concentrations were highly heterogeneous, whereas spectrophotometric measures of purity were generally stable, though A260/230 revealed occasional deviations (Table 3). Research conducted in China by Qiu, *et al*., 2021 showed that 12 MIRU-VNTR, in addition to representing a low workload, has good discriminatory power for this population[29]. Other studies conducted in Iran and India such as Chatterjee & Mistry, 2013 and Jafarian, *et al*., 2010 demonstrated low discriminatory power for these populations, recommending that 12 MIRU-VNTR be used in conjunction with Spoligotyping, or that the use of more polymorphic loci be carefully evaluated for certain study populations[30,31]. Other research by Rasoahanitralisoa, *et al.*, 2017 and Yin, *et al.,* 2023 suggested increasing the number of loci to 1532,33 and, other authors such as Oelemann, *et al.,* 2007; Sann, *et al.*, 2020 mention that both 15 and 24 locus MIRU-VNTR typing showed similar discriminatory power demonstrating the applicability of this technique for population-based studies of MTB transmission[34,35].

Sublineage Indo-Oceanic lineage 1 has not been reported in Ecuador; however, a study conducted by Balcells, *et al.*, 2015 in Chile reports its existence[36]. Another study published by Woodman, *et al.*, 2019 reports the existence of this sublineage in Latin American countries such as Mexico, Brazil, Chile and Venezuela[37]. The existence of this sublineage has also been reported in Colombia and Peru, which makes its presence in Ecuador possible due to the commercial exchange and migration between these countries.

On the other hand, lineage 4 in studies conducted between 2013 and 2016 by Garzon, *et al.*, 2020, show this lineage as the most prevalent in Ecuador, with 98.4% for a total of 373 study samples[38]. Within this lineage is the S sublineage, which in the afore mentioned study reported a prevalence of 13.1%. Furthermore, in another study conducted by whole genome sequencing in 2019 in Quito, Ecuador by Zurita, *et al.*, 2019, the Latin American and Mediterranean (LAM) lineage was reported to be the most prevalent[39]. A study investigating the transmission dynamics of TB in a coastal Ecuadorian province bordering Colombia analysed 105 strains of the *Mycobacterium tuberculosis* complex using 24-loci MIRU-VNTR and spoligotyping, and found exclusively Lineage 4[40].

The study results are in concordance with Maghradze, *et al*.[5,] who mentioned that MIRU 26, MIRU 10 and MIRU 16 are among the MIRU with the highest allelic diversity and are more relevant when discriminating the sublineage or lineage to which a study sample belongs. The discriminatory value of MIRU-VNTR loci is based on the established Hunter–Gaston index, as reported by Maghradze, *et al.* (2022) and others[4]. In this study, MIRU 16 showed allelic variation (2, 4, and 6 repeats across three samples), suggesting marker variability, though the limited sample size prevents firm conclusions.

Although molecular epidemiology has not been widely used in Ecuador for TB control, this study highlights its potential by employing strain typing and lineage and sublineage determination using 12 MIRU-VNTR loci using genetic material extracted from sputum smears. This approach may improve understanding of how delays in TB diagnosis and treatment affect outcomes in Latin America, a problem documented in multiple South American countries[18,41,42].

Limitations. The analysed slides corresponded to individuals could differ from the broader population in the studied area. The 12-loci set has reduced resolution compared to 15/24-loci, which could limit the differentiation of closely related strains and overestimate clustering. Limited DNA yield, possible degradation, and PCR inhibitors may have reduced MIRU-VNTR efficiency and hindered lineage determination.

Conclusions

Genotyping by 12-MIRU-VNTR from positive ZN-s allows the identification of Indo-Oceanic Lineage 1, followed by Euro-American Lineage 4 from primary care settings. A larger sample is needed to confirm lineage prevalence and fully assess genotyping directly from stored positive ZN-s to improve tuberculosis control.

Author contributions

The following authors Stalin Bedón, Bryan Cevallos, Alexis Naula, Héctor Javier Sánchez-Pérez, Natalia Romero-Sandoval, Patricia Jiménez-Arias participated in conceptualisation, methodology, software, validation, formal analysis, research, resources, data curation, writing-preparing the original draft, writing-revising and editing, visualisation, supervision, project management, acquiring funding. All authors have read and approved the published version of the manuscript.

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