Study of the distribution of lineages of *Mycobacterium tuberculosis* in a prison in Guayaquil, Ecuador

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Summary

The lineages of *Mycobacterium tuberculosis,* the causative agent of tuberculosis, a highly contagious infectious disease in Ecuador, have not been determined in prison conditions. Therefore, the purpose of this study is to establish for the first time the lineages prevalent in a prison population in Guayaquil, Ecuador. 36 clinical isolates obtained from Persons Deprived of Liberty (PDL) at the Male Social Rehabilitation Center No. 1 in Guayaquil during 2016 were analyzed. The clinical isolates belong to the collection of the Mycobacteria Reference Laboratory of the National Institute of Public Health Research (INSPI) of Guayaquil. The genotypes for each isolate were determined by MIRU-VNTR (15 loci), performing individual PCR. The highest prevalence 97.2% (35/36) corresponds to the Euro-American lineage 4 made up of sub-lineages: LAM (58.2%), Haarlem (16.7%), Ghana (16.7%) and S (5.6%); while the 2 East Asian lineages represented 2.8% (1/36) corresponding to the Beijing sub-lineage. A high prevalence of the Euro-American lineage 4 is observed, mainly the LAM sub-lineage with the presence of MDR-TB. Being a confinement environment, it is vital to improve the diagnosis, treatment, and epidemiological surveillance strategies along with genotypic ones.

Key words:
Tuberculosis. Mycobacterial interspersed repetitive units. Genotyping. Lineages. Surveillance. MDR-TB.

Estudio de la distribución de linajes de *Mycobacterium tuberculosis* en una cárcel de Guayaquil, Ecuador

Resumen

Los linajes de la tuberculosis, una enfermedad infectocontagiosa altamente prevalente en Ecuador no ha sido determinada. El propósito de este estudio es establecer los linajes prevalentes en la cárcel de hombres de la ciudad de Guayaquil, Ecuador. Se analizaron 36 aislados clínicos obtenidos de personas privadas de libertad del Centro de Rehabilitación Social Varones No. 1 de Guayaquil durante el 2016. Los aislados clínicos pertenecen a la colección del Laboratorio de Referencia de Micobacterias del Instituto Nacional de Investigación en Salud Pública e Investigación (INSPI) de Guayaquil. Los genotipos para cada aislado se determinaron mediante MIRU-VNTR (15 loci), realizando PCR individuales. La prevalencia más alta 97,2% (35/36) corresponde al linaje 4 euroamericano integrado por los sub-linajes: LAM (58,2%), Haarlem (16,7%), Ghana (16,7%) y S (5,6%); mientras que el linaje 2 East Asian representó el 2,8% (1/36) correspondiente al sub-linaje Beijing. Se observa una alta prevalencia del linaje 4 euroamericano principalmente el sub-linaje LAM con presencia de MDR-TB. Al ser un ambiente de confinamiento es vital mejorar las estrategias de diagnóstico, tratamiento y vigilancia epidemiológica junto con genotípica.

Palabras clave:
Tuberculosis. Unidades repetitivas intercaladas de micobacterias. Genotipificación. Linajes. Vigilancia.

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Introduction

Tuberculosis (TB) is an infectious contagious disease, considered among the top 10 causes of death worldwide and the main one due to a single infectious agent *Mycobacterium tuberculosis*. The risk of infection depends on both, endogenous factors of the bacteria and exogenous factors[1]. However, the probability that an infected person will develop the disease is determined by the state of general health, nutritional, and immunocompetence[2]. Besides, multi-drug resistant TB is a health threat and a challenge because of the need for more toxic and expensive drugs[3].

In 2020, 1.5 million people died from TB, including 214,000 among persons living with HIV and an estimated 10 million developed the disease[4]. The World Health Organization (WHO) estimated 282,000 new and recurring cases of TB in the Region of the Americas in 2017, 3% of the global burden of TB (10 million cases), and an incidence rate of 28 per 100,000. population. In the Americas, the highest incidence rate was observed in the Caribbean (61.2 per 100,000 inhabitants), followed by South America (46.2), Central America and Mexico (25.9), and North America (3.3)[5].

In Ecuador, the data shown for 2018 reflected that TB caused 460 deaths in HIV negative people and 210 deaths in HIV positive people. The prevalence of pulmonary TB is approximately 60 cases per 100,000 people. In this year 7,200 people developed the disease and there was an incidence rate of 44 per 100,000 inhabitants[6].

In persons deprived of liberty (PDL) the incidence of TB is higher, reaching up to 100 times compared to the general population. The main factors contributing to this situation are overcrowded conditions in cells, poor ventilation, limited access to health care, socio-economic level of PDLs, low-level education, and little information about TB[7]. Additional factors are alcohol and other substance addictions, as well as comorbidities such as hepatitis or HIV infection[8]. Increased incidence of TB is directly associated with the frequency of previous arrests and the length of incarceration[9]. According to the Pan American Health Organization (PAHO) in Ecuador, in the period 2016-2017, a TB incidence rate was reported in prisons of 1,674 per 100,000 inhabitants and a relative risk of TB in PDL of 50.7%, being the second-highest value registered in the region and surpassed only by El Salvador (99.0%)[5]. TB is not an inevitable consequence of incarceration and can be controlled through programs based on WHO's proposed global TB management strategy.

Molecular epidemiology is a very useful tool to improve the understanding of the biology of pathogenic bacteria, virulence, and transmissibility. Genotyping is a method used to trace specific isolates of *Mycobacterium tuberculosis* in a community and study the genetic diversity and transmission dynamics of the disease[10]. Philip Supply in 2005 proposed a genotyping system based on variable-number tandem repeats (VNTRs) of mycobacterial interspersed repetitive units (MIRU), these are microsatellites, with a size between 40 and 100 bp in size and are present in the species of the *Mycobacterium tuberculosis* complex[11]. MIRU-15 is a fast and efficient technique in the discrimination and allocation of epidemiological clusters of *M. tuberculosis* isolates. It is a reliable method for the evaluation of epidemiological and phylogenetic studies, which is why it is considered as the gold standard for genotyping[12].

The MTBC lineages have a high phylogeographical population structure, with some lineages and sublineages existing globally and others exhibiting strong geographical restriction, leading to the conclusion that the strain types are uniquely suited to distinct human populations[13]. Consequently, there are seven *Mycobacterium tuberculosis* complex (MTBC) lineages that are dispersed globally and classified as either modern or ancestral. Lineage 1 (Indo-Oceanic), Lineage 2 (East Asia), Lineage 3 (India-East Asia), Lineage 4 (Euro-American), Lineage 5 (West African 1), Lineage 6 (West African 2) and Lineage 7 (Ethiopia). The most significant sublineages are as follows: lineage 1, MANU and EAI; lineage 2, Beijing; lineage 3, Central Asian (CAS) and Delhi; lineage 4, Haarlem (H), Latin American Mediterranean (LAM), T, X, S, Ghana, URAL, TUR, Uganda, and H37Rv; and lineage 6, AFRI and West African[14,15].

The objective of this study was to identify and report for the first time the lineages and sub-lineages of *M. tuberculosis* circulating within a microenvironment such as the men's prison in the city of Guayaquil, Ecuador.

Material and method

Clinical isolates

Thirty-six clinical isolates belonging to the *Mycobacterium tuberculosis* complex obtained from PDLs at the Male Social Rehabilitation Center No. 1 in Guayaquil were analyzed during 2016. The clinical isolates belong to the collection of the Reference Laboratory of Mycobacteria of the National Research Institute in Public Health and Research (INSPI) of Guayaquil. The reference strain *Mycobacterium tuberculosis* H37Rv was used as a control.

Extraction of DNA

M. tuberculosis isolates were resuspended in Tris HCl-EDTA and heat-inactivated at 80ºC for 20 minutes, followed by DNA puri-

fication and precipitation according to the phenol-chloroform standardized protocol[16].

MIRU-VNTR technique

The genotypes for each isolate were determined after combining the results of the 15 loci in the following sequence: 580 (MIRU4), 2996 (MIRU26), 802 (MIRU40), 960 (MIRU10), 1644 (MIRU16), 3192 (MIRU31), 424 (Mtub04), 577 (ETRC), 2165 (ETRA), 2401 (Mtub30), 3690 (Mtub39), 4156 (QUB4156), 2163b (QUB11b), 1995 (Mtub21), and 4052 (QUB26)[12] performing individual PCR for each loci, the amplified fragments were analyzed using 2% agarose gels and Image Lab software was used to evaluate the amplicon sizes automatically (Bio-Rad, USA). Allelic allocation was performed according to the proposed methodology[17]. The H37Rv strain was used to validate the results for a specific locus by comparing them to the allele number assigned to that locus.

Genotyping data analysis

On the MIRU-VNTRplus platform (http://www.miru-vntrplus.org/), phylogenetic analysis of clinical isolates and lineage assignment to each isolate were performed by comparing the mirutypes obtained from the isolates under study with the available database. In order to generate a phylogenetic tree of unordered pairs of arithmetic means (UPGMA Tree) and clonal complexes with a minimum spanning tree (MSTree), the maximum MIRU-VNTR locus difference within a clonal complex was set to 2[18,19].

Beijing Lineage Identification by Allele-Specific PCR (ASO-PCR)

The identification of a Beijing lineage isolate via comparative analysis on the MIRU-VNTR plus platform was confirmed by the identification of the Beijing lineage-specific SNP via Allele-Specific PCR (ASO-PCR). The SNPs C114A (Rv0102) and C2565T (Rv2839) were used to confirm whether an isolate belonged to the Beijing or Non-Beijing lineage[20].

Results

The isolates belong to young PDL with an average age of 33 years, 38.89% of the cases correspond to an age range between 18 and 30 years. Thirty isolates out of thirty-six (83.33%) are new cases of TB, 5.56% (2/36) relapses and 11.11% (4/36) correspond to PDL who were in treatment at the time of taking of samples.

Regarding drug sensitivity profiles, 19.44% (7/36) presented resistance to isoniazid, 8.33% (3/36) resistant to streptomycin, 5.56% (2/36) multidrug-resistant (MDR-TB), 5.56% (2/36) are polyresistant isolates to isoniazid and streptomycin simultaneously and 61.11% (22/36) are sensitive to drugs. There are three PDLs co-infected with HIV, of which one strain is MDR-TB.

Analysis of genotypes on the www.miru-vntrplus.org platform identified 5 sub-lineages: LAM 58.2% (21/36), Ghana 16.7% (6/36), Haarlem 16.7% (6/36), S 5.6% (2/36) and Beijing 2.8% (1/36) (Figure 1). The Beijing sub-lineage was confirmed by allele-specific oligonucleotide PCR (ASO-PCR) and has no drug resistance.

One hundred percentage (7/7) of the isoniazid-resistant isolates were associated with LAM sub-lineage. The streptomycin-resistant strains are distributed in the sub-lineage Ghana 66.67% (2/3) and LAM 33.33% (1/3). The Haarlem and S sub-lineages include polyresistant strains combined with isoniazid and streptomycin with one case for each. The 2 TB-MDR isolates are located in the Haarlem and LAM sub-lineages respectively. The strains of people coinfected with HIV were associated with the LAM sub-lineage 66.67% (2/3) and Ghana 33.33% (1/3). Three cases of two strains were identified sharing an identical mirutype, two in the LAM sub-lineage, and one in the Ghana sub-lineage.

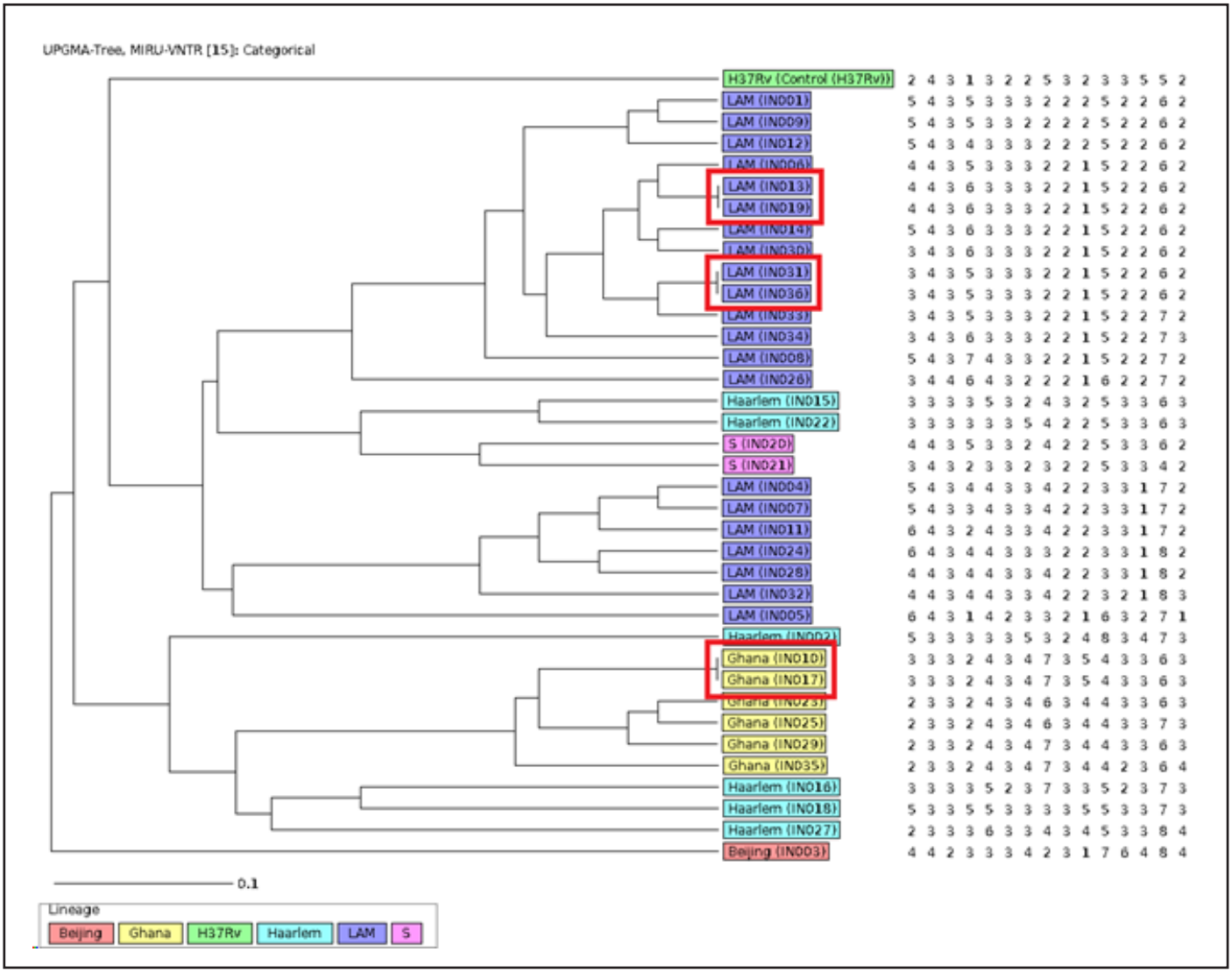
In the molecular analysis of clustering through www.miru-vntrplus.org, 24 isolates (66.67%) were organized in 3 clonal complexes (CC1, CC2, and CC3) with 12 isolates for CC1 and 6 isolates for clonal complexes CC2 and CC3 respectively, and 12 (33.33%) noclustered isolates named as orphan isolates. CC1 and CC2 clustered the isolates corresponding to the LAM lineage, which is the predominant lineage found in the study and, presumably, the most transmissible in the prison setting. CC3 clustered the Ghana lineage isolates, which are more dispersed due to phylogeographic differences. In addition, 12 isolates were discovered that did not belong to any clonal complex and were therefore deemed singletons. These are believed to be infections outside of prison or reactivations of the disease (Figure 2).

Discussion

This is the first MTB genotyping study carried out in a prison (Male Social Rehabilitation Center No. 1 in Guayaquil) in Ecuador with the aim to identify the prevalent lineages and sub-lineages, drug resistance patterns, and know the transmission dynamics of the disease inside the prison.

Thirty-six clinical isolates were characterized by molecular methods using the 15 loci MIRU-VNTR technique and the highest prevalence 97.2% (35/36) corresponds to the lineage 4, Euro-American, made up of sub-lineages: LAM (58.2%), Haarlem (16.7%), Ghana (16.7%) and S (5.6%); while the lineage 2, East Asian, representing 2.8% (1/36) corresponding to the Beijing sub-lineage. Studies carried out in other Latin American countries

Figure 1. UPGMA phylogenetic tree of 36 clinical isolates of *Mycobacterium tuberculosis* from the Male Social Rehabilitation Center No. 1 in Guayaquil based on 15 loci MIRU-VNTR. The red boxes indicate the strains that have the same mirutype.



such as Brazil, Venezuela and Peru have reported high prevalence for the LAM and Haarlem sub-lineages[21-23] as well. In Ecuador, studies carried out on samples from hospitals report prevalence of 92%, 33.7% and 17.1% for the LAM sub-lineage 17.1%[24,25], this means that both within and outside of the prison, the circulating sub-lineages with the highest prevalence are LAM and Haarlem. It is important to mention that MDR-TB isolates are found within these prevalent sub-lineages.

Rueda VG[20]. and Grandjean *et al.*[22] reported that the LAM sub-lineage presents a high percentage of resistance to one or more first-line antibiotics used for the treatment and prevention of TB; these authors suggest that *M. tuberculosis* strains belonging to this sub-lineage could have compensatory mutations against resistance-giving mutations, which would allow them greater adaptation and replication ability while developing resistance to antibiotics[26]. Zurita *et al*. reported that the LAM sub-lineage presented a greater number of cases of resistance to isoniazid, this coincides with what is reported in this study[25].

Research carried out in 2016 by Reis *et al.*[7] in Brazil, showed a rate of isolates mono resistant to isoniazid similar to our study, which suggests the problems associated with the use of this drug. These findings show that the resistance acquired by LAM may be related to difficulties in the management of TB, abandonment of treatment, and lack of epidemiological surveillance.

In different studies, it has been documented the great virulence and the antibiotic resistance of *Mycobacterium tuberculosis* stra-

ins belonging to the Beijing sub-lineage[27,28]. However, there are cases where it is sensitive to all antibiotics used for TB treatment like the one we present in this study[29]. Liu *et al*.[26] mention that the difference in the results obtained regarding antibiotic resistance for this sub-lineage is related to the variety of Beijing genotypes and the percentage of modern and ancient genotypes of lineage 2. The resistance or sensitivity of the Beijing lineage could be linked to the variation in the treatment regimens, compliance with the protocols, and the variable quality of the drugs used[31].

It is important to mention that MIRU-VNTR, which is considered the gold standard for genotyping, is a reliable method for assessing epidemiological and phylogenetic studies, however techniques using next-generation sequencing, such as whole-genome sequencing (WGS), have recently been gaining importance because they provide the most comprehensive source of information on the genoma content of a given clinical isolate. However, MIRU-VNTR remains a valuable method for *M. tuberculosis* genotyping, particularly in low-income countries, as WGS requires adequate bioinformatic support and specialised expertise, and is expensive[32,33].

Molecular genotyping is a very useful tool when studying epidemic events and the transmission dynamics of the disease; In this study, it allowed us to identify 3 cases with an epidemiological link due to having an identical mirutype. Regarding the drug sensitivity patterns of these epidemiological links, we found that of isolates 10 and 17, isolate 10 presented resistances to streptomycin, while isolate 17 was sensitive to said antibiotic; isolates 13 and 19 they share the same susceptibility test profile and in isolates 31 and 36, isolate 36 presents resistance to isoniazid, while isolate 31 is sensitive to this antibiotic. The 6 isolates mentioned correspond to new cases of TB.

Puerto *et al*.[23] mention that by characterizing the clinical isolates, it is possible to identify cases of people who share a *Mycobacterium tuberculosis* strain with an identical genotype, which is highly associated with the same transmission route, although the link may be indirect, whereas Kato-Maeda *et al.*[10] propose that isolates nonclustered or unique genotypes are considered the product of reactivation of latent infection, presumably acquired outside the prison population. In our case, the isolates with an epidemiological link due to sharing the same genotype are clustered into clonal complexes 1 and 3, which makes us think that the contagion could have occurred within the prison facilities. Nonclustered isolates considered "singletons" correspond to cases of reactivation of the disease (Figure 2).

Regarding coinfection with HIV, in this study, the existence of one case of MDR-TB coinfection with HIV is reported. The PAHO mentions that the presence of this coinfection represents an important challenge in the prevention, diagnosis, and treatment of both diseases. Firstly, because of the progression from HIV to AIDS in presence TB and, with it, possible death. And for the promotion of clinical evolution of the disease in people infected with *Mycobacterium tuberculosis* with a high rate of recurrence, extrapulmonary forms, and mortality due to a decrease in CD4 lymphocytes counts from HIV[5].

It is estimated that TB patients coinfected with HIV are 67% more likely to develop monoresistant, polyresistant, multidrug-resistant, and extremely resistant TB compared to people with TB who are not HIV positive[34]. Is important to highlight that this clinical isolate belongs to an MDR-TB prisoner coinfected with HIV, it is a new case and may be due to infection within the facilities of the social rehabilitation center.

Droznin J, *et al*.[28] mention that, although MDR-TB can arise when patients do not complete a full course of anti-tuberculosis drugs, it can be transmitted from person to person through infected droplets in the air even to those who have never taken anti-tuberculosis medicines or been in contact with another person with TB. This makes MDR-TB a particular concern in crowded and closed areas, such as prisons. Different studies show high mortality among HIV-infected MDR-TB patients compared to uninfected ones and alarming mortality among HIV-infected and XDR-TB patients that occurs within a few weeks after diagnosis[36].

Of the total of the cases analyzed in this study, 83.33% are primary infections. One could think that the PDLs were infected with the *Mycobacterium tuberculosis* and developed the disease within the facilities of the prison, also, 5.56% of the cases correspond to relapses in the disease, this reflects the problems that exist with the management of TB and the deficiencies in the treatment of the disease.

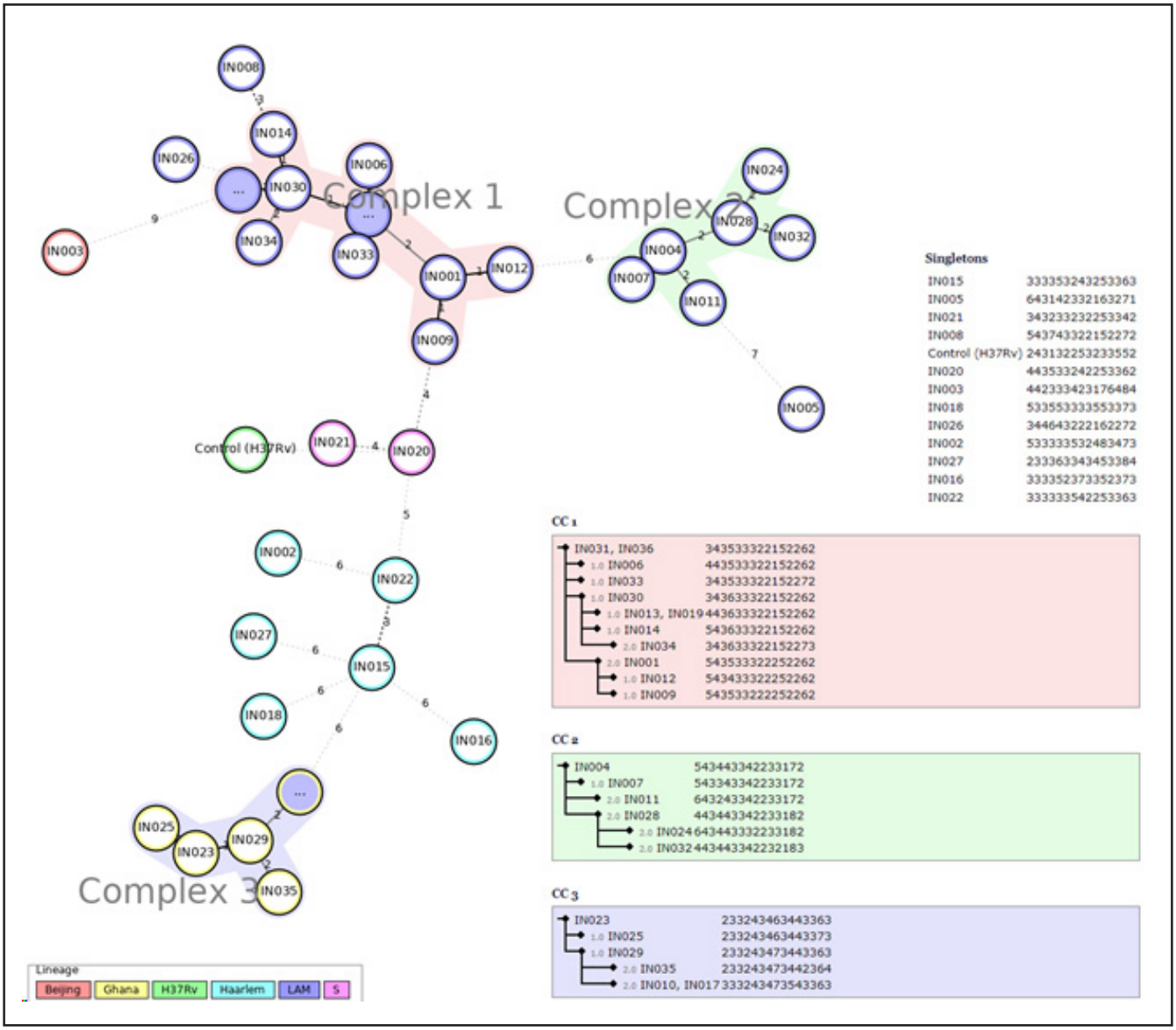
As stated by Reis *et al*.[7], the control of TB in prisons is a priority and includes the active search for respiratory symptoms, timely diagnosis, and treatment under the Directly Observed Treatment, Short-course (DOTS) strategy.

The limitations of this study were the reduced number of clinical isolates and the difficulty of access to the clinical information of the PDL and their history of admission or re-entry to prison.

Conclusions

It is the first genotyping study carried out in a prison in Ecuador in which a high prevalence of the Euro-American lineage 4, mainly the LAM sub-lineage, is observed. The presence of MDR-TB clinical isolates in a confined environment is a concern and obliges health authorities to strengthen active search strategies, timely diagnosis, treatment under the DOTS strategy, and epide-

Figure 2. Minimal expansion tree, clonal complexes and singletons clinical isolates of 36 *Mycobacterium tuberculosis* isolates from the Male Social Rehabilitation Center No. 1 in Guayaquil based on 15 loci MIRU-VNTR.



miological surveillance using molecular tools in all the country's prisons. Such as the one described above, this study gives us more information on the transmission dynamics of TB and allows us to make timely decisions.

Author contribution

Sandoval-Romero N, Jimenez A: Study conceptualization and design, data interpretation, manuscript writing/editing. Cifuentes L, Garcés E: data analysis and manuscript writing/editing. Garcés E and Jiménez P: laboratory work development. Greta F: critical review of dataset and samples. All authors critically reviewed the manuscript.

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Conflict of interest

The authors do not declare a conflict of interest.

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