Country-Wide Rapid Screening for the *Mycobacterium tuberculosis* Beijing Sublineage in Ecuador Using a Single-Nucleotide Polymorphism-Polymerase Chain Reaction Method

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Abstract

**Background:** Strains of the Beijing sublineage of *Mycobacterium tuberculosis* have caused large outbreaks of tuberculosis, often involving multidrug resistance strains and this genetically highly conserved family of strains predominates in some geographic areas. For most of the countries of Latin America, no country-wide studies about the prevalence of the Beijing lineage are available. **Methods:** In this study, we determine the prevalence of the Beijing sublineage in Ecuador, using a large nation-wide sample of 991 isolates from the years 2014–2016 and with the strains, in case-related-proportional representation, emerging from most of the provinces of the country. The isolates were genotyped with a single-nucleotide polymorphism (SNP) polymerase chain reaction for the Beijing sublineage. SNP-positive strains were confirmed as belonging to this lineage with 24 mycobacterial interspersed repetitive unit variable number of tandem repeat and DNA sequencing. **Results:** We identified only four Beijing isolates in this collection of 991 strains and calculated a prevalence rate of 0.43%. **Conclusions:** Our study shows a limited dissemination of the Beijing strains in the Ecuadorian population. This in contrast with the neighbor countries of Peru and Colombia were locally a prevalence of up to 16% has been reported.

**Keywords:** Beijing family, Ecuador, single-nucleotide polymorphism, tuberculosis

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**Introduction**

Tuberculosis (TB) remains a major global health problem and one of the most important infectious diseases worldwide. According to the WHO, an estimated 10.4 million people fell ill with TB, and there were over 1.5 million deaths caused by TB.1 In a global effort to reduce the TB burden, molecular epidemiology approaches based on genotypic analysis of *Mycobacterium tuberculosis* (MTB) isolates have been developed to identify transmission clusters and for a better understanding of the virulence factors and the transmission dynamics of this pathogen, necessary for its potential eradication.2,3 Using genotyping methodologies, population structure (lineage and clusters) for MTB on different locations can be established. Among the MTB lineages, the Beijing family belonging to East Asian lineage has been considered one of the most dangerous pathogens due to selective advantages compared to other MTB lineages, such as an increased capacity for transmission and drug resistance.

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to acquire drug resistance, an increased transmissibility, and hypervirulence.[4,5] Evolutionary studies have shown that the Beijing family emerged 6600 years ago in the northeast region of China, Korea, and Japan, and migration movements have expanded this strain around the world, with the prevalence ranging from over 40% in East Asia to up to 10% in the Americas.[3,6]

Although there are many reports about the molecular epidemiology of Beijing lineage in areas with a high prevalence, there is still a gap in knowledge regarding this topic in South America. In general, the reported prevalence for Latin American countries ranks from 0% to around 5%.[7-10] with the exception of several studies in Peru where prevalence rates up to 16.4% have been reported. The spread of Beijing lineage has been associated with a strong Chinese and Japanese migration to Peru during the last century.[3,11-14] Another study in Southeast Colombia reports a prevalence of 15.6% for the Beijing lineage.[15] In general, all these studies in South America are focused on a single location without addressing the question of the country-wide prevalence. For Ecuador, using mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) typing, we recently reported a low prevalence of 1.6% for this MTB lineage despite the fact of having open border with Peru and Colombia, where the highest rates of Beijing lineage in South America have been reported.[16] However, our study has limitations: only a small sample of strains (n = 380) were used to determine this prevalence and only MIRU-VNTR was used to identify Beijing strains. The aim of the present study was to confirm the low prevalence of Beijing lineage in the Ecuadorian population based on a country-wide screening and to exclude the presence of local Beijing clusters. In our first study, we used MIRU-VNTR typing for the detection of the Beijing lineage in a strain collection. This technique is laborious and relatively expensive. Here, we use a single-nucleotide-polymorphism detection polymerase chain reaction (SNP-PCR), a rapid and relatively cost-effective method for the detection of the Beijing lineage.[17,18] We used this method with a collection of 991 strains emerging from a representative part of the Ecuadorian population, and we included all the samples available at the time of this study for the provinces of Guayas, Pichincha, and Cañar where Beijing MTB strains have been previously reported.[16] This collection included 394 strains resistant to isoniazid and/or rifampicin, and we confirm an even lower prevalence of the Beijing genotype in our country and lack of clustering of the Beijing strains as we have reported previously.[16]

**Materials and Methods**

**Mycobacterium tuberculosis isolates**

A sample of 991 MTB strains, including 373 isolates earlier typed with MIRU-VNTR[16] and isolated from pulmonary and extrapulmonary TB patients in the years 2014–2016, were used in this study. This collection included all the strains inactive and stored for research purpose from the years 2014–2016 available at the “Laboratorio Nacional de Referencia para Micobacterias” from “Instituto Nacional de Salud Publica e Investigacion” (INSPI) in Quito and Guayaquil. These laboratories are the only public laboratories in Ecuador that confirm TB cases with culture on Lowenstein–Jensen medium. Besides, these laboratories perform drug resistance testing for the first- and second-line TB antibiotics for most of the MTB cultures.

**Microbiology of the Mycobacterium tuberculosis strains**

Culture and susceptibility testing were performed by an experienced laboratory staff, using standard methods, approved by the Pan-American Health Association. All strains with a drug resistance profile were tested for resistance to rifampicin, isoniazid, streptomycin, ethambutol, and pyrazinamide. Drug resistance information for the strains is summarized in Table 1.

**Mycobacterium tuberculosis heat inactivation and DNA isolation**

MTB isolates were resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid) and inactivated by boiling at 95°C for 45 min. Inactivated samples were centrifuged for 5 min at 10,000 g, and the supernatant was directly used for genotyping, without the use of a specific DNA extraction procedure, as has been previously reported.[16,19]

**Beijing lineage identification of Mycobacterium tuberculosis isolates**

The identification of the Beijing lineage was done with a SNP-specific PCR protocol, as has been described previously.[17-21] More specifically, we performed a SNP-PCR protocol developed to detect the Beijing lineage-specific SNP (Rv2154724 [G526A]). This SNP has been used before in a publication that described the first TB case in Ecuador infected with a Beijing strain.[21] The “in-house” PCR protocol for the detection of this SNP used two PCR reactions for each MTB isolate. The reverse primer (5’-CTGACACCGATGAAATTCGC-3’) is the same for both reactions, whereas in one reaction, the forward primer includes the SNP at the 3’ end (5’-CTGCTGGCCCAGGAAGG-3’) and in the other reaction, the forward primer (5’-CTGCTGGCCCAAGGAA-3’) does not include the SNP at the 3’. This last PCR reaction was run for the detection of non-Beijing lineage isolates.

| Drug resistance | Number of isolates, n (%) |
|-----------------|--------------------------|
| **RIF**         | 61 (7.9)                 |
| **ISO**         | 129 (16.8)               |
| **MDR**         | 68 (8.9)                 |
| **STR**         | 76 (9.9)                 |
| Ethambutol      | 11 (1.4)                 |
| PIR             | 24 (3.1)                 |
| Susceptible for all drugs tested | 551 (71.9) |

No drug resistance profile was available for 163 strains. MDR: Multidrug resistant, PIR: Pyrazinamide, ISO: Isoniazid, RIF: Rifampicin, STR: Streptomycin
Detection of Beijing isolates with the single-nucleotide-polymorphism detection polymerase chain reaction product

PCR products were separated on a 2% agarose gels and visualized with SYBR-safe DNA staining under ultraviolet irradiation. Criteria for considering an isolate positive for Beijing were a ratio of >1 for the band intensity of the Beijing-specific PCR compared to the PCR band of the non-Beijing-specific PCR. Beijing-negative and Beijing-positive controls were included in each DNA electrophoresis. The DNA band intensity was analyzed with ImageJ software, U. S. National Institutes of Health, Bethesda, Maryland, USA, an open-source image-processing program.

Confirmation of the Beijing lineage

Strains selected as Beijing positive were genotyped with the 24-MIRU-VNTR typing technique.[16,22,23] Lineages were assigned for these isolates by comparing their MIRU-VNTR patterns with those present in the MIRU-VNTRplus platform (http://www.miru-vntrplus.org). In addition, for all the isolates, positive for Beijing lineage with the SNP-PCR, the presence of the specific mutation was also confirmed with Sanger DNA sequencing using the PCR product of the primer pair forward 5’-CTGACACCCGATTTCGC-3’ and reverse 5’-CTGCTGCAGCGCCAGA-3’.

Ethical committee approval

The Ethical Committee of the University San Francisco de Quito approved this investigation with code number 2017-023IN.

Results

Our sample of 991 strains emerged from 22 of the 24 provinces in Ecuador. This collection corresponded with a heat-inactivated strain collection for the years 2014–2017 [Table 2]. The strains represented approximately 17% of the TB cases yearly diagnosed in Ecuador (5815 in the year 2017) and about 17% of the strains that were isolated or sent for drug resistance testing to INSPI-Guayaquil in this 3-year time period (5738 strains). Among the 991 isolates tested with the SNP-PCR, 62 isolates (6.3%) did not yield any detectable band in the agarose DNA electrophoresis and were excluded from the study. As to the residual 929 strains, 766 strains had information on drug resistance and 258 strains (33.6%) were resistant to isoniazid and/or rifampicin. Table 1 summarizes the details and drug resistance percentages for other drugs. SNP-PCR resulted negative for 919 samples, and 10 samples showed a band intensity of the PCR product that could be considered positive for the Beijing-specific SNP. These ten strains were genotyped with 24-MIRU-VNTR. In addition, we sequenced the region around the Beijing-specific SNP with the primer pair described in the materials and methods section. Only four of the ten isolates were confirmed as belonging to the Beijing lineage with both DNA sequencing and 24-MIRU-VNTR analysis [Table 3]. SNP-PCR allows us to calculate a prevalence of 0.43% (4 out of 929 strains) of the Beijing lineage in the Ecuadorian population. Table 3 resumes the epidemiological information for the four Beijing lineage isolates. Two are isolates from the year 2014 and the other two are from 2015. Three isolates came from sputum samples, and one strain was isolated from a urine sample. Concerning the location, two strains corresponded to the Andean region of Ecuador, the provinces of Pichincha (Quito) and Azogues. The other two strains came from the coastal region of Ecuador, from the most populated city of the country, Guayaquil. The two isolates from Guayaquil are multidrug-resistant (MDR) TB strains, whereas the other two isolates were susceptible for all the first-line TB antibiotics [Table 3].

Discussion

Among the MTB lineages, Beijing family is considered among the most dangerous due to its high virulence and transmissibility and its association with drug resistance.[5,4,24] In the Americas, Peru and Colombia have registered a high prevalence of 16.4% and 15.6%, respectively, of Beijing strains in selected areas.[3,5,11-15] Ecuadorian has open borders with Colombia and Peru, which are areas of intense trade and human migration because these countries are members of the Andean Community, and no passport is required to cross borders. In this scenario, transmission of Beijing strains from Colombia and/or Peru could be a potential threat and challenge for the national TB control program.

The first report of a Beijing isolate in Ecuador dates from 2017[18] and the second, a study from our hands, is from

Table 2: Distribution of Mycobacterium tuberculosis isolates included in the study across the provinces of Ecuador

| Coastal region | Number of isolates | Andean region | Number of isolates | Amazonian region | Number of isolates |
|----------------|--------------------|---------------|--------------------|------------------|--------------------|
| Los Rios       | 61                 | Tungurahua    | 2                  | Morona-Santiago   | 1                  |
| Guayas         | 592                | Azuay         | 8                  | Sucumbios         | 13                 |
| Esmeraldas     | 28                 | Chimborazo    | 4                  | Orellana          | 1                  |
| El Oro         | 35                 | Cotopaxi      | 3                  | Pastaza           | 2                  |
| Manabi         | 24                 | Bolivar       | 2                  | Napo              | 4                  |
| Santa Elena    | 16                 | Cañar         | 18                 | Zamora-Chimichepe | 2                  |
| Santo Domingo  | 13                 | Pichincha     | 86                 |                  |                    |
| Total          | 769                | Loja          | 1                  |                  | 23                 |
| Total          |                    |               |                    |                  | 124                |

The provinces are grouped in the three distinct geographical regions of Ecuador: Coastal region, Andean region, and Amazonian region. One MTB isolate, from the Galapagos Islands and 74 other MTB isolates, with no clear region identification, are not included. MTB: Mycobacterium tuberculosis
2019.\textsuperscript{21} Both studies analyzed a limited amount of strains, and no country-wide prevalence data were reported in these studies. We reported a low prevalence of 1.6% of the Beijing lineage using solely 24 MIRU-VNTR typing, detecting six Beijing strains in a convenience sample of 373 country-wide distributed isolates.\textsuperscript{16} Following reviewer’s suggestions concerning our previous study, we decide to implement a SNP-PCR-based detection method\textsuperscript{14} to search for Beijing strains in the whole collection of 991 strains available at Instituto Nacional de Salud Publica e Investigacion. (INSPI), including the 373 strains previously typed with de MIRU-VNTR technique. SNP-PCR has been used to determine the prevalence of Beijing and other MTB lineages in different epidemiological contexts.\textsuperscript{17,20,21} With this method, we preselected ten strains of which four were confirmed as Beijing with MIRU-VNTR typing and sequencing of the SNP. These four strains were coincidentally already reported as Beijing in our previous study; thus, the SNP technique did not alter the number of strains identified as Beijing for the 991 strains tested. Of particular is that two strains, detected with MIRU-VNTR typing in our previous report, were not detected with the SNP technique. However, a deeper characterization of these 373 strains by regions of difference (RD) genotyping and whole-genome sequencing (WGS) confirmed that only the four strains identified as Beijing by SNP-PCR and sanger sequencing in the present study were correctly genotyped as Beijing (manuscript under preparation). We, therefore, consider the SNP technique of great value as a first screening method for the detection of Beijing and estimate its sensitivity in 100% compared to 24 MIRU-VNTR + RD. However, the specificity of the SNP test has its limitation. Only four out of the ten strains preselected with this method were real Beijing strains, so the protocol implemented in this study would be helpful for areas with middle-to-low prevalence of Beijing strains.

Regarding Beijing lineage prevalence in Ecuador, we confirmed an ultra-low prevalence, estimated with SNP-PCR, of 0.43% in agreement with our previous report.\textsuperscript{16} Hence, the epidemiological scenario for Beijing lineage in Ecuador is the same described for most of the countries in South America, with a low prevalence rate compared to other MTB lineages.\textsuperscript{7-10} So far, although there is an increasing trend for Beijing lineage spreading in the neighboring country of Peru, with reports showing an increasing prevalence from 5.5% in 2005 to 16.4% in 2015,\textsuperscript{13} our data show that Peruvian Beijing strains are not able to spread across the Ecuadorian border.\textsuperscript{16} In addition, despite a high prevalence up to 15.6% of Beijing lineage in the city of Buenaventura in Colombia, relatively close to the border with Ecuador, no Ecuadorian Beijing isolates were found in a province of the north border of Ecuador. Altogether, these findings challenge the consensus of the ultra-adaptive and expansive features for Beijing MTB strains, as has previously being reported for other drug-resistant MTB strains in Latin American countries.\textsuperscript{8}

Our four Beijing isolates showed phylogenetic clustered association in pairs,\textsuperscript{16} correlating with their geographical location; two of them in Guayaquil and other two in the Andean region as has been previous reported.\textsuperscript{16} While the two isolates of the Andean region of Ecuador lack first-line antibiotic resistance, the two isolates from Guayaquil were MDR-MTB strains. It is important to notice that in the present study, 592 MTB isolates were from Guayas province, where the highest prevalence of TB has been registered in the capital of this province, Guayaquil, the most populated city in Ecuador. In our previous study, we analyzed only 164 MTB isolates from this province. This strongly supports the lack of micro-epidemics due to MDR Beijing isolates in this part of Ecuador.

In addition, our results show no strong association between drug resistance and the Beijing genotype in Ecuador as also has been found for Peru, where this genotype has little influence on the epidemiology of MDR and extensively drug-resistant-TB.\textsuperscript{14} This is in contrast with Valle del Cauca in Colombia where the Beijing genotype represented 15.6% of the isolates and correlated with MDR-TB.\textsuperscript{15}

Regarding the limitations of this study, we do not totally exclude the micro-epidemics of the Beijing strains in the Andean provinces where this genotype has been detected because we could only increase the sample size in this study, compared to the previous study,\textsuperscript{16} from 5 isolates to 18 isolates and from 62 isolates to 86 isolates for Cañar and Pichincha provinces, respectively.

**Conclusions**

We confirm an extremely low prevalence and lack of active transmission of MDR Beijing strains in Ecuador, particularly in the city of Guayaquil, with the highest TB burden in Ecuador. Although in the Andean region the Beijing isolates were not associated with drug resistance, we cannot exclude other risk factors associated with these strains like hypervirulence; hence,
our research group will develop studies in these provinces to look for possible micro-epidemics. In addition, WGS sequencing with our Beijing isolates is ongoing, which will provide us with deeper insight into the particular features of the Beijing strains of Ecuador.

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Conflicts of interest
There are no conflicts of interest.

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