Probable long-term prevalence for a predominant Mycobacterium tuberculosis clone of a Beijing genotype in Colon, Panama

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Abstract

Beijing genotype Mycobacterium tuberculosis strains associate with increased virulence, resistance, and/or higher transmission rates. This study describes a specific Beijing strain predominantly identified in the Panamanian province of Colon with one of the highest incidence of tuberculosis in the country. Retrospective Mycobacterial Interspersed Repetitive-Unit/Variable-Number of Tandem Repeats analysis of 42 isolates collected between January-August 2018, allowed to identify a cluster (Beijing A) with 17 (40.5%) Beijing isolates. Subsequent prospective strain-specific PCR based surveillance from September 2019 to March 2020, confirmed the predominance of the Beijing A strain (44.1%) in this province. Whole genome sequencing revealed higher-than-expected diversity within the cluster, suggesting long-term prevalence of this strain and low number of cases caused by recent transmission. The Beijing A strain belongs to the Asian African 3 (Bmyc13, L2.2.5) branch of the modern Beijing sublineage, with their closest isolates corresponding to cases from Vietnam, probably introduced in Panama between 2000 and 2012.

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Summary

Beijing genotype *Mycobacterium tuberculosis* strains associate with increased virulence, resistance, and/or higher transmission rates. This study describes a specific Beijing strain predominantly identified in the Panamanian province of Colon with one of the highest incidence of tuberculosis in the country. Retrospective Mycobacterial Interspersed Repetitive-Unit/Variable-Number of Tandem Repeats analysis of 42 isolates collected between January-August 2018, allowed to identify a cluster (Beijing A) with 17 (40.5%) Beijing isolates. Subsequent prospective strain-specific PCR based surveillance from September 2019 to March 2020, confirmed the predominance of the Beijing A strain (44.1%) in this province. Whole genome sequencing revealed higher-than-expected diversity within the cluster, suggesting long-term prevalence of this strain and low number of cases caused by recent transmission. The Beijing A strain belongs to the Asian African 3 (Bmyc13, L2.2.5) branch of the modern Beijing sublineage, with their closest isolates corresponding to cases from Vietnam, probably introduced in Panama between 2000 and 2012.

Keywords

Mycobacterium tuberculosis , Beijing lineage, strain-specific-PCR, predominant, Panama

Introduction

The *Mycobacterium tuberculosis* (MTB) complex may be subdivided into seven lineages (Comas et al., 2013) with unequal geographic distribution. Among them, the Beijing lineage (Lineage 2), has received greater attention because of its successful global distribution, increased transmissibility and virulence, and involvement in major outbreaks in some settings (Bifani, Mathema, Kurepina, & Kreiswirth, 2002; Borrell et al., 2009; Iwamoto et al., 2012; Yang et al., 2012). Certain Beijing clones have been associated to resistance and higher ability to compensate for the loss of fitness linked to the acquisition of drug resistance.

The Beijing lineage constitutes around 13% of the global MTB complex population (Parwati, van Crevel, & van Soolingen, 2010). Since its discovery in East Asia in 1995 (van Soolingen et al., 1995), many studies have documented its endemic prevalence in Asia (˜50% of the circulating strains in East Asia) (Parwati et al., 2010), as well as its presence in South Africa (27%) (Gandhi et al., 2014) and northern Eurasia (40-60%) (Mokrousov, 2013). However, limited information is available on its representativeness in other geographic

areas worldwide, e.g., the Caribbean and Central America, were an average of 3.5 % of total tuberculosis (TB) cases in the area associated to the Beijing lineage have been reported (Millet, Baboolal, Streit, Akpaka, & Rastogi, 2014) and some more specific data about its presence (2.1%) in Guatemala, where the Beijing lineage was involved in a severe outbreak (Saelens et al., 2015) .

The purpose of this study was to complete a preliminary information (Dominguez et al., 2019) on the presence of this lineage in Panama. We focused our efforts on Colon, a Panamanian province with the highest TB incidence rate (MINSA, 2018). Based on a modular strategy that combined Mycobacterial Interspersed Repetitive-Unit/Variable-Number of Tandem Repeats (MIRU-VNTR)-based typing (Supply et al., 2006), strain-specific PCRs, and whole genome sequencing (WGS) we aim to i) update the data on Beijing strains in Colon, ii) evaluate the role of Beijing strains in recent transmission active events, and iii) explore the Beijing phylogeny and history in Panama.

Materials and Methods

Mycobacterium tuberculosis isolates

All 110 MTB clinical isolates collected (January 2018-March 2020) in the province of Colon, Panama, were selected. A sample of clinical isolates from the province of Chiriqui was obtained from Hospital Obaldia (Panama) as the comparison group. Isolates were cultured on Lowenstein–Jensen medium and DNA extracted using the cetyl trimethylammonium bromide method (van Soolingen, Hermans, de Haas, Soll, & van Embden, 1991).

Allele-specific oligonucleotide polymerase chain reaction (ASO-PCR)

Specific targeted alleles and PCR conditions for Beijing*Mycobacterium tuberculosis* strain A (Beijing A) were as described elsewhere (Dominguez et al., 2019). In brief, PCR reaction mixtures included a final volume of 25 µl: 1.25 µl of 5 U/µl HotStarTaq® DNA polymerase (Qiagen, Hilden, Germany), 0.4 µl of 25 mM MgCl2, 2.5 µl of 10x PCR buffer with 15 mM MgCl2, 5 µl of 5x of Q solution, primer pool concentration (2.5 µl mix: 2µM, 6 µM, 5 µM), 0.2 µl of 25 mM dNTPs, water, and the DNA target. PCR conditions were as follows: 95 ºC for 15 min, followed by 27 cycles of 95 ºC for 1 min, annealing 64 ºC for 1 min, and 72 ºC for 10 min. The ASO-PCRs were performed on the isolates from September 2018 and July 2019 in Madrid (Spain) and the technique was transferred to Colon (Panama) for prospective *in situ* application on the new incident cases (August 2019 to March 2020).

Genotyping and whole genome sequencing

Isolates were genotyped by MIRU-VNTR, based on a 24 loci-set described elsewhere (Supply et al., 2006). An additional set of four hypervariable loci was added for the Beijing isolates (Allix-Beguec et al., 2014). WGS on the Illumina MiSeq platform, mapping, and single nucleotide polymorphism (SNP) calling was performed as detailed elsewhere (Dominguez et al., 2019). The median-joining network was constructed from the SNP matrix generated for each case using the NETWORK 5.0.0.1 program Sequences were deposited in EMBL-EBI database (Accession numbers PRJEB39699, PRJEB23681 and PRJEB29408).

Phylogenetic analysis

For general phylogenetic purposes, we compared MIRU-VNTR types against those (> 8,000 isolates) in general global MIRU-VNTR datasets and specific Beijing databases (Merker et al., 2015; Mestre et al., 2011).

For the phylogenetic analysis, WGS reads were aligned to the *M. tuberculosis* H37Rv (NC_000962.3) genome sequence with Bowtie 2 (Langmead & Salzberg, 2012). SAMtools v.0.1.18 and FreeBayes v.1.1.0 were used for variant calling (Koboldt et al., 2012; Marth, 2012). For FreeBayes, SNPs with a minimum mapping quality of 20, minimum coverage of 10 and alternate fraction of 0.9 were taken.

SNPs from our WGS data were compared against global NGS collections (> 9,000 downloaded MTB genomes) (Shitikov et al., 2017). For more specific analysis within the Beijing lineage, we compared WGS

data against 200 strains corresponding to the L2.2.5/Asian African 3 subgroup (Supplementary Table). The phylogenetic tree was built based on overall SNPs after excluding repetitive, mobile elements, PE-PPE-PE_RGRS, drug-resistance associated genes, and artifact SNPs linked to indels using RAxML v8.2.11 (Stamatakis, 2014) under a GTR-CAT model with ascertainment bias correction. A subset of genomes of the L2.2.3/Asia Ancestral sublineage was taken as an outgroup. A smaller subset (clade including the Panama and Vietnamese isolates) was used for aligning and identifying the 858 SNP positions. Bayesian analysis of molecular sequences (BEAST) of three independent chains of 100 mio iterations (using evolutionary models: GTR, molecular clock, UCLD, population mode, and GMFR, Skyride) was performed. Three chains converged after about two mio iterations (considered burn-in and removed) and were combined into a single dataset that was then used to construct the maximum clade credibility tree.

Results

A retrospective MIRU-VNTR analysis of 42 MTB isolates collected in Colon (January-August 2018) initially revealed that 40.5% (17/42) belonged to a major cluster of pansusceptible Beijing strains (cluster Beijing A). Most isolates (12/17) shared an identical 28-loci MIRU-VNTR pattern, and four different single-locus variants (SLVs) were detected (two of them involving a hypervariable locus), represented by 1, 2, 1 and 1 isolates (Table 1). Only four of the remaining non-Beijing isolates were clustered and distributed in different families: Haarlem (23.8%), H37RV-like (16.7%), LAM (16.7%), and S (2. 4%).

Next, a prospective sample of MTB isolates from 68 incident cases in Colon (September 2018-March 2020) was analysed, applying a Beijing A-specific PCR. The band-pattern expected for the Beijing A strain was obtained in 30 isolates (44.1%).

To assess whether the Beijing A strain -highly represented in Colon- was also present in other regions in the country, we selected another Panamanian province, Chiriquí (278 Km from Colon) and applied strain-specific PCR on a sample of 21 incident cases (cases from 2019). No amplification was obtained for two cases and the Beijing A strain pattern was not detected in any of the 19 amplified isolates.

Thirty Beijing A isolates, retrospectively and prospectively identified, for which appropriate quality DNA was available, were analyzed by WGS, and 25 of them offered enough coverage depth (>20X) to perform SNP calls, from which a network of relationships was built (Figure 1). We detected noticeable SNP-based diversity, most isolates with long pairwise genetic distances and distributed along different branches. The maximum SNP-distance was 38 and the median shortest SNP distance between two strains was five. Five not-sampled nodes (median vectors) were detected. Some small subgroups within the network shared shorter pairwise genetic distances (2-7 SNPs) and only two pairs of isolates showed 0 SNPs between them. Only one of the isolates with SLVs in MIRU-VNTR offered enough coverage and occupied an independent branch in the network (Figure 1).

Phylogenetic Analysis

We first compared the data of the 24 loci MIRU-VNTR against that of MIRU-VNTR*plus*(https://www.miru-vntrplus.org/MIRU/index.faces). The most frequent pattern found for the Beijing A strain was MLVA type 1048-33, while the less common patterns with SLVs (Table 1) were MLVA types 1048-32 and 342-33 (Table 2). We next searched for these three MLVA types in a compiled 24-MIRU-VNTR global dataset of 4,987 Beijing isolates (Merker et al., 2015) and found that MLVA type 1048-33 was scarce, with only five entries, and remarkably two of were from Panama; 1048-32 was more frequent, 158 isolates widely distributed in Asia and fewer entries from Latin America; 342-3 was poorly represented (12 isolates) (Table 2).

WGS-analysis revealed that Beijing A cluster corresponds to the modern Beijing sublineage, specifically its Asian African 3 (Bmyc13; L2.2.5) sublineage (Figure 2). The entries showing relatedness with Beijing A strains in our study matched Vietnamese isolates (Figure 2). We selected the currently publicly available sequences of MTBC Beijing subgroup L2.2.5/Asian African strains (˜ 200 strains) to construct a maximum likelihood phylogeny that grouped, again, Colon Beijing A representatives with strains isolated in Vietnam (Ho Chi Minh City), with a good bootstrap support (96%).

Finally, (Figure 3) we focused on a smaller subset corresponding to the clade that included the Panama Beijing A isolates and related Vietnamese isolates to determine their convergence at a time point between 2000-2012 by BEAST analysis. The resulting median substitution rate was estimated to be 4.0 x 10ˆ-7 [95% HPD 2.75 x 10ˆ-7 - 5.11 x 10-7], which corresponds to 1.6 subs/genome/year (95% confidence interval 1.1 - 2.0/year).

Discussion

Due to its key location at the isthmus between North and South America, Panama has undergone great demographic and population changes of diverse geographic origins, which has probably affected the composition of circulating MTB strains. Few and discontinuous efforts have been made to genotypically characterize MTB isolates, mainly on convenience samples (Rosas et al., 2013; Sambrano et al., 2014).

To help solve the lack of a systematic molecular epidemiology program in Panama, we introduced (Dominguez et al., 2019) a novel strategy as follows: i) fast MIRU-VNTR-based snapshots of the most prevalent strains, ii) targeted WGS on these major clones, and iii) tailored PCRs to identify marker SNPs for prevalent strains (Dominguez et al., 2019). This allowed us to determine that six strains were responsible for 50% of MTB cases in Panama City and Colon. These six strains were unequally distributed between the two settings, and remarkably, the Beijing lineage was mainly restricted to Colon with an incidence of 21.5%. As the Beijing strain found in Colon is pansusceptible it went unnoticed in a study carried out in Panama that focused only on multidrug-resistant (MDR) TB cases (Rosas et al., 2013). A single previous study carried out in Panama City, in which genotypic data were obtained from non-selected strains from one outpatient clinic reported a much lower percentage (3.7%) of the Beijing lineage (Sambrano et al., 2014).

The purpose of this study was to perform a more in-depth analysis of Beijing strain in the province of Colon. A standard characterization by MIRU-VNTR on a small retrospective sample of isolates alerted us that the true figures were higher than those observed from our previous convenience sample. In fact, over 40% of Colon´s isolates in 2018 were Beijing A strains. Furthermore, additional prospective labelling efforts with strain-specific PCRs, confirmed its high representativeness (44.1%) in incident cases in 2019 in the same city and its absence in a distant region in the same country. Fortunately, most of Beijing A isolates were pansusceptible with 4.8% rifampicin-resistant isolates.

Our first interpretation of the results was that the high presence of Beijing A strains was due to uncontrolled recent transmissions in Colon. Several studies have described the Beijing lineage as responsible for major outbreaks (Bifani et al., 2002; Iwamoto et al., 2012; Johnson et al., 2006; Perez-Lago et al., 2019). Colon has elevated rates of poverty, overcrowded and substandard quality housing, and high HIV coinfection, all factors associated to active MTB transmission. However, WGS analysis of 26 Beijing A isolates revealed higher-than-expected SNP-based diversity, beyond diversity thresholds (12 SNPs) (Walker et al., 2013) for determining transmission clusters, with few cases showing SNP based distances consistent with recent transmissions.

Our findings confirm the homoplasy described before in MIRU-VNTR typing, particularly for the Beijing lineage (Alaridah et al., 2019; Gurjav et al., 2016; Nikolayevskyy, Kranzer, Niemann, & Drobniewski, 2016), revealed by the high discriminatory power of WGS, as described for other settings (Gardy et al., 2011; Stucki et al., 2016). Moreover, our data allows to suggest that the Beijing A strain has probably been present for a long time, with only a minority of cases due to recent transmission.

This new long-term status for the Beijing A strain, as revealed by WGS data, led us to redirect our efforts from our initial molecular epidemiological purpose, namely, to track recent transmissions for their control to another phylogenetically-oriented interest, i.e. to try to explain the reasons behind the predominance of the Beijing A strain in Colon. For this, we first needed to identify the geographical origin for this strain.

Comparisons of the MIRU-VNTR and SNP profiles against global datasets placed the Beijing A strain to the Asian African 3 branch of the modern Beijing sublineage (L2.2.5), which is mainly reported in East Asia (Luo et al., 2015; Merker et al., 2015; Shitikov et al., 2017). The modern Beijing sublineage is considered more virulent and with a higher mutation rate than the ancestral Beijing (Yang et al., 2012). Further detailed

analysis of our WGS data positioned the Beijing A representatives close to isolates from a sublineage isolated in Vietnam, with strong bootstrap support (96%). This suggests that the origin of the Colon Beijing A strain may be traced in Southeast-Asia, probably from a non-predominant strain, as scarce L2.2.5 (0.7%) presence in studies with good strain representativeness from Vietnam (Mestre et al., 2011) has been reported. The estimated substitution rate per genome and year for the Beijing A strain is 1.6, which is high when considering the average substitution rates in MTB. Eldholm *et al* . determined a slightly lower substitution rate in a similar dataset (85 Beijing isolates), still consistent with the normally elevated substitution rates observed throughout the Beijing lineage (Eldholm et al., 2016).

Global spread of the Beijing lineage results from decades of migration events. The introduction from Liberia to the island of *Gran Canaria* (Spain) in 1993, responsible for its fast subsequent spread in the Canary Islands (Perez-Lago et al., 2019) and its importation to the USA from Asia (Bifani et al., 2002), are examples of recent events. In Peru, the Beijing strain is detected in 9% of TB cases in certain settings; its introduction in this Latin American country probably occurred during Chinese immigration in the mid-19th century for economic and political reasons (Iwamoto et al., 2012). Similarly, the most obvious historical event that may explain the entry of an inedit MTB lineage in Panama is the massive migration from abroad to assure the workload required for the construction of the Panama Canal, although migrants were mainly from France, China and India ("Sanitation and the Panama Canal," 1905), rather than from Vietnam. In addition, our analysis indicates that the Beijing A strains converged at a time point between 2000 – 2012, while the most recent ancestor between the Panama and Vietnam strains has a time interval of 1987 – 1995. Thus, the Beijing A strain must have been introduced into the region sometime after 2000 (most likely just before 2012), far more recently than the construction of the Canal in the 19^{th} century, and before the more recent Canal expansion in 2015.

Panama´s geographical location at the isthmus between North and South America draws people from many different origins. Most (˜85%) people of the Panamanian population have Asian, European and American descendants/ancestors (Arias TC, 2002). Immigration to Panama from Asian countries has been increasing over the years. Between 2000 and 2012 over 50,000 passengers were reported to enter Panama from Asian countries every year ("Instituto Nacional de Estadísticas y Censo, Republica de Panamá (INEC). Movimiento internacional de Pasajeros (2000-2012),"). Thus, the introduction of the Beijing A strain may have easily occurred in any of these multiple opportunities rather than during a previous major historical event.

In summary, to overcome the lack of a systematic molecular epidemiology program in Panama we used a combined MIRU-VNTR and strain-specific PCR approach that allowed us to identify high presence of the Beijing lineage in a specific Panamanian setting. The analysis based on WGS data were key to reveal the long-term nature for this lineage and rule out a role uncontrolled recent transmission. Phylogenetic analysis suggests a not too remote importation from Southeast Asia of a not well represented isolate form the Modern Beijing sublineage. The precise reasons behind the introduction of this Beijing strain and spread throughout the province of Colon requires classical epidemiology and in-depth-omic analysis of both *M. tuberculosis* and human population.

Figures

Figure 1: Network of relationships for *Mycobacterium tuberculosis*Beijing A isolates from Panama based on whole genome sequencing data. Each dot represents a single-nucleotide polymorphism. When two isolates are in the same box, they show no single-nucleotide polymorphism between them. mv, median vector corresponding to not-sampled nodes; REF: Most recent common ancestor. $^{+}$ Isolate with single-locus-variant (SLV) as per the MIRU-VNTR analysis (the remaining isolates with SLVs did not offer enough coverage in the whole genome sequencing analysis for single-nucleotide polymorphism calls). *isolate collected in 2015.

Figure 2**.** Phylogenetic reconstruction of Panamanian Beijing isolates. Maximum-likelihood phylogeny inferred from 5,809 variable single-nucleotide positions of the 26 isolates rooted with the H37Rv (NC_000962.3) reference strain. The most recent common ancestor "genome" was constructed and compared against modern and ancestral Beijing strains datasets.

Figure 3. Maximum clade credibility tree (BEAST) to infer the estimated circulation time of the Beijing strain in Panama of Panama clade groups (26 isolates) with strains isolated in Vietnam (Ho Chi Minh City) and the British Columbia (most likely foreign-born from South-East Asia). Posterior probabilities of nodes are in grayscale (ranging from 0.0 – 1.0). Node ages with 95% HPD ranges are indicated as blue bars.

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Conflict of interest: No author declare conflict of interest.

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Figure 1.