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Research paper

Genotypic diversity of *Mycobacterium tuberculosis* in Buenos Aires, Argentina

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ABSTRACT

Buenos Aires is an overpopulated port city historically inhabited by people of European descent. Together with its broader metropolitan area, the city exhibits medium tuberculosis rates, and receives migrants, mainly from tuberculosis highly endemic areas of Argentina and neighboring countries. This work was aimed to gain insight into the *Mycobacterium tuberculosis* population structure in two suburban districts of Buenos Aires which are illustrative of the overall situation of tuberculosis in Argentina. The Lineage 4 Euro-American accounted for > 99% of the 816 isolates analyzed (one per patient). Frequencies of spoligotype families were T 35.9%, LAM 33.2%, Haarlem 19.5%, S 3.2%, X 1.5%, Ural 0.7%, BOV 0.2%, Beijing 0.2%, and Cameroon 0.2%. Unknown signatures accounted for 5.3% isolates. Of 55 spoligotypes not matching any extant shared international type (SIT) in SITVIT database, 22 fitted into 15 newly-issued SITs. Certain autochthonous South American genotypes were found to be actively evolving. LAM3, which is wild type for RD^{rio}, was the predominant LAM subfamily in both districts and the RD^{rio} signature was rare among autochthonous, newly created, SITs and orphan patterns. Two genotypes that are rarely observed in neighboring countries– SIT2/H2 and SIT159/T1 Tuscany– were conspicuously represented in Argentina. The infrequent Beijing patterns belonged to Peruvian patients. We conclude that the genotype diversity observed reflects the influence of the Hispanic colonization and more recent immigration waves from Mediterranean and neighboring countries. Unlike in Brazil, the RD^{rio} type does not play a major role in the tuberculosis epidemic in Buenos Aires.

1. Introduction

Curbing tuberculosis (TB) depends on rapid diagnosis, effective therapy, and control of transmission. The introduction of genotyping strategies in the characterization of clinical *Mycobacterium tuberculosis* isolates has enabled major advances in our understanding of the transmission dynamics of this pathogen (Jagielski et al., 2016). The systematic incorporation of these strategies into health program activities has allowed identifying risk factors for transmission, designing tailored public health interventions, and assessing the success of control measures.

Over the years, different techniques have been used to study transmission dynamics. These techniques include the restriction fragment length polymorphism using the insertion sequence IS*6110*, PCR-based techniques like spoligotyping, variable number tandem repeats of mycobacterial interspersed repetitive units, and large sequence polymorphisms (Jagielski et al., 2016). Single nucleotide polymorphism barcode approaches based on high throughput whole genome technology are probably the most accurate tools currently available to classify clinical isolates and construct high resolution and reproducible phylogenies. However, until data produced by such technologies become widely available, the analysis of information based on simpler genotyping approaches can still cast light on the genotypic structure of *M. tuberculosis* in as yet insufficiently explored areas (Brudey et al., 2006; Midori Kato-Maeda et al., 2011; Sola et al., 2001).

Globally, seven *M. tuberculosis* lineages have been described, with different phylogeographic specificities. These are Lineage 1 Indo-Oceanic (which corresponds to East African Indian or EAI lineage in the

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Table 1
Distribution of the 816 *M. tuberculosis* isolates included in the study according to district and period.

District	Population[a]	TB cases per 100,000 inhabitants[b]	Culture-positive TB cases per 100,000 inhabitants†	Study period	N° of isolates analyzed	Proportion of culture-positive TB cases in the district	Participating centre
Buenos Aires Metropolitan Area, North	292.878	25.7	14.6	Jul 2002–Jun 2012	225	53%	Hospital Central de San Isidro
Buenos Aires Metropolitan Area, West	1.775.816	39.8	18.1	Jul 2002–Dec 2011	591	19%	Hospital de Agudos Paroissien, Centro de Zoonantroponosis de La Matanza

[a] Source: 2010 census, http://www.sig.indec.gov.ar/censo2010/

[b] Source: Rates are calculated as averages of the study period based on http://www.anlis.gov.ar/iner/wp-content/uploads/2016/11/Notificacion-de-casos-de-Tuberculosis-en-la-Republica-Argentina-Periodo-1980-2015.pdf.

SITVIT database), Lineage 2 East-Asian (including Beijing), Lineage 3 East-African-Indian (corresponding to Central Asian or CAS in SITVIT database), Lineage 4 Euro-American, Lineage 5 West Africa (*M. africanum* I), Lineage 6 West Africa (*M. africanum* II), and Lineage 7, more recently described in the Horn of Africa (Gagneux et al., 2006; Hirsh et al., 2004). The vast majority of strains circulating in Latin America belong to Lineage 4 Euro-American, assumedly brought from Europe during the European exploration and colonization. It comprises several families, namely Latin American-Mediterranean (LAM), the ill-defined T superfamily, Haarlem (H), X and S, as defined by spoligotyping (Jagielski et al., 2016).

Argentina is a fairly large South American country with wide disparities in terms of physical geography, ethnicity, socio-economic development, and TB rates. Since 2003, our National Reference Laboratory has put emphasis in the characterization of drug-resistant (Monteserin et al., 2017) and, most particularly, multi-drug and extensively drug resistant (MDR/XDR) clinical isolates of *M. tuberculosis* countrywide. Strain genotyping showed that more than a half of MDR/XDR isolates are included in seven major clusters, each circumscribed within a defined geographic area (Ritacco et al., 2012). Our preliminary, non-systematic, observations have suggested that the spectrum of susceptible *M. tuberculosis* genotypes circulating in the country is more diverse than that of MDR/XDR-TB strains. However, a comprehensive study of the *M. tuberculosis* population structure in Argentina has not yet been performed. Within this context, the present study was aimed to explore the genetic diversity of *M. tuberculosis* strains circulating in the Metropolitan Area of Buenos Aires, which is fairly illustrative of *M. tuberculosis* diversity in Argentina. Indeed, near half of all ~10,000/yr incident TB cases in the country are diagnosed in this urban area, which is inhabited by over one-third of the country's population and is also the seat of its main seaport.

2. Materials and methods

2.1. Population

The Buenos Aires Metropolitan area (or Greater Buenos Aires) includes the port city of Buenos Aires and the surrounding urban area. This latter consists in several overpopulated suburban districts, which together present higher rates of poverty and extreme poverty (33% and 7%, respectively, at the time of the study) than the average rates for the country. They also host recent migrants who originate mainly, but not exclusively, from highly TB endemic areas of Argentina and neighboring countries and use to pay frequent visits to their places of origin. We analyzed 816 clinical *M. tuberculosis* isolates obtained in the period July 2002–June 2012 in three centers located in two of these suburban districts, namely San Isidro and La Matanza, representative of low and high degrees of unsatisfied basic needs, respectively. During the study period, annual TB rates in San Isidro (31.7 to 19.9 per 100,000) followed closely the national trend, while in La Matanza, rates fluctuated amply (24.4 to 50.4 per 100,000), probably owing to variations in the efficiency of case-finding activities. In the present population-based study, we included all available isolates of patients (one isolate per patient) with new and previously treated culture-positive TB consecutively diagnosed in the participating centers during the study period. The Hospital Central de San Isidro is a 160-bed general hospital providing microbiological diagnosis and care to ~320 patients suspected of having TB per year, of which ~25 result confirmed bacteriologically. The Hospital Interzonal General de Agudos Paroissien is a 315-bed regional hospital and the Centro de Antropozoonosis is a district outpatient clinic located in La Matanza. Together, they provide bacteriological analysis to ~1000 patients suspected of having TB per year, of which ~70 result confirmed bacteriologically. Even though the estimated coverage of our study approached 90% of culture-confirmed TB patients diagnosed in the participating institutions, the samples represent 53% and 19% of all culture-confirmed TB cases reported in the

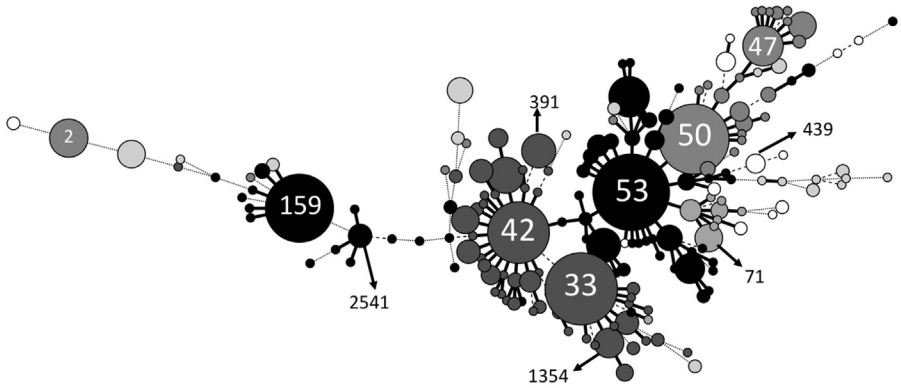

Fig. 1. A minimum spanning tree illustrating evolutionary relationships between the 816 spoligotypes in the study.
The length of the branches represents the distance between patterns. The number of allele/spacer changes between two patterns are denoted as follows: up to 1 change, solid black line; up to 3 changes solid gray line; up to 5 changes dashed gray line; up to 7 o more changes dotted gray line. The size of the circle is proportional to the total number of isolates in our study.

period in San Isidro and La Matanza districts, respectively (Table 1). As these districts, particularly La Matanza, extend over a wide geographic area and the cases missed in this study were out of the influence of the participating health centers, we assume the sample to be truly representative of the core study population. Suppl. Table 1 describes demographic, clinical and microbiological characteristics of the study population. Ethics committee approval and informed consent were not required because the work was retrospective and medical records were anonymized prior to analysis.

2.2. Microbiological studies

All specimens were cultured on Löwenstein-Jensen slants. Species identification was performed according to standard procedures. On the basis of programmatic guidelines, *M. tuberculosis* isolates submitted to drug susceptibility testing were only those obtained from patients considered to be at risk for drug resistance. Drug susceptibility testing to first- and second-line drugs was performed at INEI-ANLIS National Reference Laboratory under supranational proficiency testing according to WHO standard (WHO, 2009).

2.3. Genotyping

DNA was obtained by boiling lysis of actively grown bacilli harvested from Löwenstein-Jensen slants. Spoligotyping was performed according to the international standard protocol (Kamerbeek et al., 1997). We compared the spoligotypes in SITVITEXTEND, which is a proprietary extension of the SITVITWEB database available at the website of the Institut Pasteur de la Guadeloupe (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) (Demay et al., 2012). Isolates with autochthonous, orphan or newly created spoligotypes of LAM, T and undefined families were submitted to a multiplex PCR designed to identify the LAM-RD^{rio} subtype through the detection of a large specific deletion (Gibson et al., 2008). We did not investigate the RD^{rio} status of geographic-specific genotypes classified as Haarlem, S, and X because these families are genetically distant from LAM and therefore are not likely to harbor the LAM RD^{rio} specific deletion. Selected isolates with marked geographic specificity were further characterized by MIRU-VNTR 24 typing (Supply et al., 2006). The MIRU-VNTR*plus* tool (http://www.miru-vntrplus.org) was applied for MIRU-VNTR similarity search assessment (Allix-Béguec et al., 2008).

We used BioNumerics version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) to compare spoligotyping patterns. We applied the SpolSimilaritySearch web-tool which is based on SITVIT2 (http://www.pasteur-guadeloupe.fr:8081/SpolSimilaritySearch/) to explore the country distribution patterns of selected spoligotypes (Couvin et al., 2017).

2.4. Statistical analysis

We used MedCalc software v9.3.6.0 (Frank Schoonjans, Belgium) for statistical analysis. A p value $< .05$ was considered statistically significant.

3. Results

3.1. Overall strain family distribution

The overall frequencies of *M. tuberculosis* families were: T 35.9% (n = 293), LAM 33.2% (n = 271), Haarlem 19.5% (n = 159), S 3.2% (n = 26), X1.5% (n = 12), Ural 0.7% (n = 6), BOV 0.2% (n = 2), Beijing 0.2% (n = 2), Cameroon 0.2% (n = 2). A total of 783 (95.9%) isolates were represented by 145 SITs (Shared or Spoligotype International Types), including 5.2% (n = 42) isolates classified in SITs with unknown (Unk) spoligotype signatures (Suppl. Table 2). Out of 55 isolates whose patterns did not match any SIT registered in the proprietary SITVITEXTEND database, 22 were classified in 15 newly created SITs after matching an orphan pattern in SITVITEXTEND or due to two or more isolates sharing a new pattern within the present study. The remaining 33 isolates displayed orphan spoligo patterns (i.e., did not match any other pattern in the SITVITEXTEND database). A total of 713 (83.4%) isolates were found to cluster in 74 SITs, ranging from 2 to 78 isolates per SIT and other 70 SITs were represented by a single isolate in this study (in addition to the 33 newly described orphan patterns). Suppl. Table 3 shows clustered SITs containing ≥10 isolates in Argentina (i.e. present study plus preexisting entries from Argentina in SITVIT EXTEND database) and their worldwide distribution according to SITVITEXTEND database.

3.2. Phylogenetic inferences

A minimum spanning tree illustrates phylogenetic relationships among the 816 *M. tuberculosis* spoligotypes included in the study (Fig. 1). Two main branches, Haarlem and LAM families, emerge from SIT53/T1, the core of the Euro-American lineage, in addition to two minor branches for S and X families. Two conspicuous clades, SIT2541/T1 and SIT159/T1 Tuscany, sprout one after the other in a single branch directly from SIT42/LAM9 (the core spoligotype for the LAM family), followed by related autochthonous orphan patterns. The MLVA MtbC15–9 type for our isolates with SIT159 is identified as 121-51 (13224433222412615332262 2) and the MIRU-VNTR type for our isolates with SIT2541 (13428433222411614333263 2) is absent from the MIRU-VNTR*plus* database. According to the MIRU-VNTR*plus* tool, both MIRU-VNTR types are close to each other (distance 0.093) and fit into the LAM family. As for more distant and sparse branches, there is insufficient evidence to support genetic relationships.

Table 2
Percentage distribution of families/subfamilies in this study as compared with distribution in five neighboring countries.

Subfamilies	Argentina	Bolivia[b]	Brazil[c]	Chile[c]	Paraguay[c]	Peru[c]
	(n:816)	(n:100)	(n:5905)	(n:591)	(n:226)	(n:3171)
Beijing	0.2	0.0	< 1	< 1	< 1	10.0
H1 and H3	16.9	37.0	11.7	13.2	17.2	29.6
H2	2.6	2.0	< 1	0.0	< 1	0.0
LAM1 and LAM2	3.1	0.0	8.8	1.2	2.6	1.1
LAM3	12.6	15.0	3.9	17.6	1.7	8.6
LAM other	17.5	11.0	27.1	17.8	45.0	14.0
S	3.2	2.0	1.8	< 1	8.3	1.0
T1 Tuscany	7.8	0.0	< 1	0.0	2.7	2.0
T other	28.1	22.0	21.5	30.8	11.8	15.0
X	1.5	0.0	3.1	3.0	< 1	4.4
Unknown	5.3	11.0	7.1	6.6	4.8	7.3
Others[a]	1.0	0.0	6.6	5.2	4.3	7.0
M. bovis	0.2	0.0	8.4	4.6	1.6	0.0

[a] Sublineages representing < 1.0% of strains were classified as « Others ».
[b] Source: Monteserin et al. (2013).
[c] Source: SITVITEXTEND database.

3.3. Compared population structure in different geographic settings

No significant, or marginal, differences were observed between San Isidro and La Matanza isolates regarding frequencies of most spoligotype families/subfamilies. Only T family strains, especially SIT159/T1 Tuscany, were significantly more present in La Matanza district (Suppl. Table 4). Table 2 shows the overall distribution of families/subfamilies in this study as compared with distribution in five neighboring countries. Haarlem strains, more precisely H1 and H3, were more abundant in Peru and Bolivia. H2 was more represented in Argentina and Bolivia than in the other countries. The most frequent LAM family in Argentina, Bolivia, and Chile was LAM3 (RD^{rio} wild). Together, LAM1 and LAM2 (RD^{rio} mutated) were substantially more frequent in Brazil than in the other countries, including Argentina. SIT159/T1 Tuscany was well represented in Argentina and to a lesser degree in Paraguay and Peru. SIT391/LAM4, a genotype hitherto reported only in Paraguay and Brazil, was herein found to be well represented in Argentina.

3.4. RD^{rio} status of spoligotypes with a geographically restricted affinity

Suppl. Table 5 presents RD^{rio} status and global country distribution of LAM, T, and Unk genotypes in our study exhibiting strongly restricted global distribution. Consecutive rows grouped by colors indicate highly related spoligotypes. Only two of the herein described new genotypes, namely 4124/LAM9 and Or12/Unk resulted mutated for RD^{rio}. In view of its RD^{rio} status, Or12 should be assigned to the LAM family. We were not able to determine RD^{rio} status of 11 isolates with new SITs or orphan types (3 LAM, 7 T, and 1 Unk) because DNAs were no longer available for testing. The remaining new SITs herein classified as LAM, T, and Unk were wild type for RD^{rio}.

Spoligotypes with strong national or regional affinity – namely SIT159/T1 Tuscany, its relative SIT2541/T1, SIT391/LAM4, SIT253/T1, and SIT1354/LAM3 – were wild type for RD^{rio}. Fig. 2 shows conspicuous examples of geographically restricted spoligotypes in this study.

4. Discussion

Upon the analysis of a large strain collection, we confirm that, as expected, the overall population structure of *M. tuberculosis* in Argentina is dominated amply by the Lineage 4 Euro-American. Altogether, the three main families in this lineage – T, LAM, and Haarlem – account for > 80% of the isolates in our study. Therefore, the distribution of genotypes in Argentina is in line with the structure described for other South American countries with similar historical roots (Lagos et al., 2016). In particular, the abundance of LAM genotypes in these countries is a reflection of the early Spanish and Portuguese colonization.

Within the worldwide spread of the LAM family, the RD^{rio} type was found to be particularly prevalent in Brazil and Portugal, and has been associated with high rate of transmission and drug resistance (Barbosa et al., 2012; Dalla Costa et al., 2013; David et al., 2012; Lazzarini et al., 2007; Von Groll et al., 2010; Weisenberg et al., 2012). Its landmark, a large deletion named RD^{rio}, was shown to be distinctly present in LAM1 and LAM2 strains and absent in LAM3 strains, whereas the remaining LAM genotypes, including the founding LAM core represented by SIT42/LAM9, may or may not present the RD^{rio} deletion. In a previous work, we found that LAM RD^{rio} type strains do not contribute substantially to the burden of drug-resistant TB in Argentina (Monteserin et al., 2017). Herein we note that LAM3 (RD^{rio} wild type) is the most frequent LAM genotype in Argentina whereas LAM1 and LAM2 (RD^{rio} mutated) are relatively infrequent. We also find that RD^{rio} type strains are barely represented among new and seemingly evolving spoligotypes in the country (Suppl. Table 4). Altogether, our results suggest that, differently from Brazil, the RD^{rio} type neither plays a major role in active TB transmission nor in potentially emerging new *M. tuberculosis* genotypes. This finding deserves further exploration because Brazil is not only bordering Argentina but is also its closest partner in terms of commercial trade and tourism.

A special comment deserves SIT391/LAM4. This genotype has hitherto been reported mainly in Paraguay and to a much lesser extent in Brazil. Herein we find it to be well represented in Argentina, thus confirming its geographic specificity for the Southern Cone of the Americas. With the input of this study, Argentina contributes with as much as 41% of all SIT391/LAM4 entries registered in SITVITEXTEND. This genotype is mainly found in La Matanza district, where a large Paraguayan community is settled. LAM391/LAM4 strains in this study are wild type for RD^{rio}, as are other LAM391/LAM4 strains from Argentina and Paraguay present in our database but not included in this study (data not shown).

Owing to its heterogeneity and ambiguity, the T superfamily is thought to provide scarce phylogenetic information. However, certain genotypes within this family do bear true identity. SIT2541/T1 is closely related to, and plausibly progenitor of, the newly created SIT4125 and two new orphan types (Or22, Or29) described herein (Suppl. Table 5). Thus, to the best of our knowledge, SIT2541/T1 could be considered another autochthonous South American genotype which is emerging and actively evolving in Argentina. Our results suggest that SIT2541 and SIT159 are closely related to each other and might derive from the LAM family.

This relationship of SIT159/T1 Tuscany with the LAM family has been previously subscribed by three independent approaches, namely polymorphisms in genes involved in replication, repair and recombination functions, MIRU-VNTR typing, and an IS*6110*-seq based classification (Abadia et al., 2010; Allix-Béguec et al., 2008; Reyes et al., 2012). In our study, SIT159/T1 Tuscany is amply represented in Buenos Aires. In addition, four new orphan genotypes (Or8, Or10, Or26, Or28, Suppl. Table 5) are closely related to it, suggesting that SIT159/T1 Tuscany, like its relative SIT2541, is undergoing active evolution in Argentina. Interestingly, more than half of all SIT159/T1 Tuscany isolates reported globally come from Argentina, which together with Austria and Italy are the only countries with SIT159/T1 Tuscany frequencies > 6% (Fig. 2). In Argentina, this genotype has been largely related to the expansion of an autochthonous strain causing MDR-TB outbreaks in two large urban areas (Aita et al., 1996; Palmero et al., 2006; Ritacco et al., 2012). As for other Latin American countries, SIT159/T1 Tuscany is relatively frequent only in Paraguay and Peru, occurs sporadically in Brazil and Mexico, and has not yet been found in Bolivia (Monteserin et al., 2013), Chile (Lagos et al.,

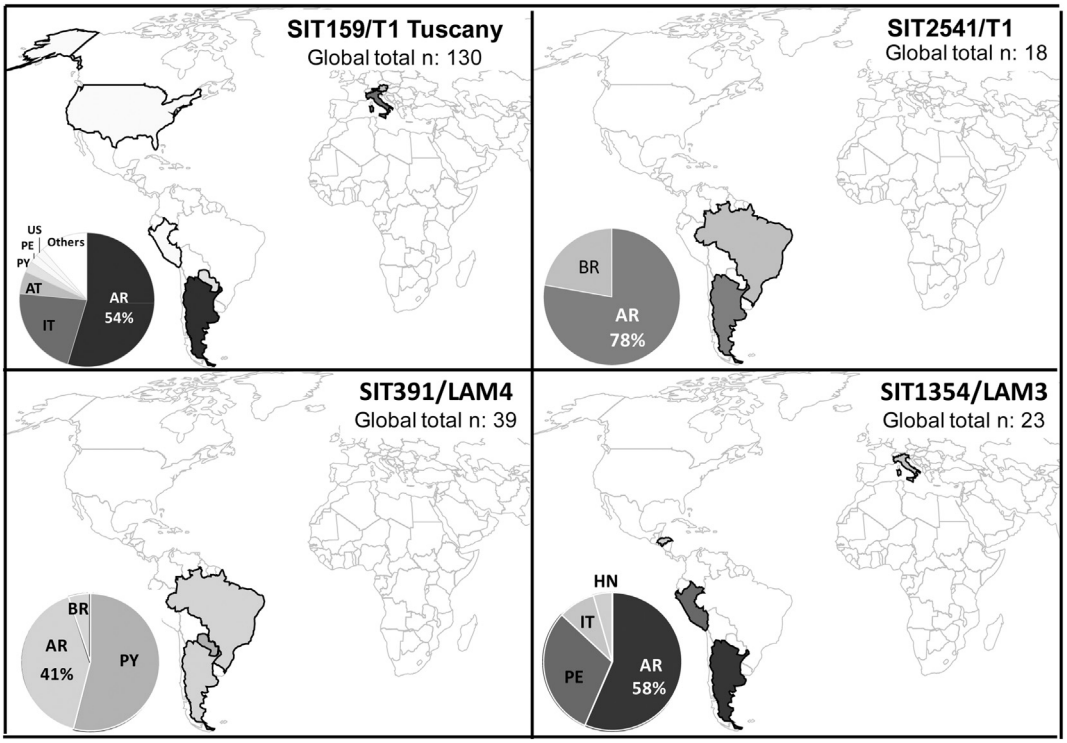

Fig. 2. Global distribution of conspicuous SITs in our study with regional specificity according to http://www.pasteur-guadeloupe.fr:8081/SpolSimilaritySearch/. AR: Argentina; AT: Austria; BR: Brazil; HN: Honduras; IT: Italy; PE: Peru; PY: Paraguay; US: United States of America.

2016), Colombia (Realpe et al., 2014), French Guiana (Millet et al., 2011), Guyana, Suriname (Streit et al., 2015), and Venezuela (Aristimuño et al., 2006). Altogether, these data reinforce the idea of the intriguingly restricted geographic specificity of SIT159/T1 Tuscany.

The long history of Italian immigration into Argentina may account not only for the presence in Buenos Aires of SIT159/T1 Tuscany but also for the presence of members of the S family (S stands for Sicily, the place where this latter genotype was first described). In fact, by the late 19th century and first half of the 20th century, Argentina hosted massive waves of Italian immigrants who settled mainly in urban areas of the central plains of the country. We find the family S not only conspicuously represented in our sample but also actively evolving, as shown by the occurrence of one new SIT (SIT4112) and three orphan spoligotypes (Or04, Or13, Or18) carrying the S signature (Suppl. Table 2).

H2 has quite distinct geographic affinities throughout the Americas. It is substantially present in the U.S.A. and the Caribbean but is infrequent in South American countries, apart from Argentina, and to a lesser degree French Guiana, Bolivia, Brazil, and Paraguay. In Argentina, it was previously found to be associated with a multidrug/extensively drug resistance and HIV infection and, as such, seems to be virtually restricted to the metropolitan area of Buenos Aires (Ritacco et al., 2012).

The Beijing family was represented in our study by two strains imported from Peru, the South American country with the highest prevalence of this family of the East-Asian lineage. Low numbers of Beijing strains, imported mainly from Peru and very occasionally from Far Eastern countries have long been lingering, but did not thrive, in Argentina (Ritacco et al., 2008). The occasional presence in Buenos Aires of two other *M. tuberculosis* genotypes that are exotic to the region – namely Cameroon and Ural – reflects the cosmopolitan nature of this port city. In turn, the finding of two *M. bovis* strains is a reminder of the zoonotic nature of bovine TB, a cattle disease which is endemic in the fertile central plains surrounding Buenos Aires. Bovine TB is steadily declining but has not yet been completely controlled in Argentina (Perezill et al., 2011). In fact, sporadic human cases due to reactivation of remote infections are expected to occur long after eradication.

This study has three major limitations. First, the study population does not cover the whole territory of Argentina. However imperfect its sampling, the work is filling a gap in the *M. tuberculosis* phylogeographic scenario in South America. Second, the study is largely based on results of spoligotyping, a tool which is vulnerable to misclassification due to convergent evolution and thus its use to infer robust phylogenetic relationships is questionable. However, the overall family strain classification based on spoligotyping has been repeatedly endorsed by more robust tools (Kato-Maeda et al., 2011; Rasoahanitralisoa et al., 2017). In particular, homoplasy is unlikely to explain a given spoligotype assignation when it is supported by strong geographic specificity, like the examples described herein. Thus, until more accurate tools are applied in further investigations, our study provides an exploratory insight into the population structure of *M. tuberculosis* in Argentina. Lastly, the exploration of RD^{rio} polymorphisms in our study was not comprehensive but directed to selected groups of interest to resolve the RD^{rio} status of particular genotypes which are potentially autochthonous to the region and/or actively evolving in Argentina.

5. Conclusions

In conclusion, as in the rest of South America, genotypes of the Lineage 4 Euro-American predominate amply in Argentina and the observed genotype diversity reflects the influence of the Hispanic colonization and more recent immigration waves from Mediterranean and neighboring countries. In contrast with the neighboring country Brazil, the RD^{rio} type does not play a major role in the TB epidemic in Argentina.

Transparency declarations.

None to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.04.006.

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