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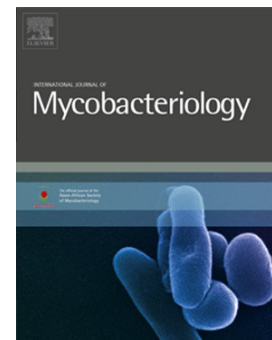
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Prevalence of Latin-American-Mediterranean genetic family in population structure of *Mycobacterium tuberculosis* in Bulgaria

Violeta Valcheva ^{a,*}, Nalin Rastogi ^b, Igor Mokrousov ^{c,*}

^a Department of Infectious Microbiology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

^b WHO Supranational Tuberculosis Reference Laboratory, Tuberculosis and Mycobacteria Unit, Institut Pasteur de Guadeloupe, Abymes 97183, Guadeloupe, France

^c Laboratory of Molecular Microbiology, St. Petersburg Pasteur Institute, 197101 St. Petersburg, Russia

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ABSTRACT

Tuberculosis (TB) control remains an important public health priority for Bulgaria. The population structure of *Mycobacterium tuberculosis* is clonal and certain genetic families of this species (e.g., Latin-American-Mediterranean [LAM]) have attracted more attention due to their global dissemination and/or particular pathogenic properties, e.g., association with multidrug resistance (MDR). The aim of this study was to evaluate the prevalence of the *M. tuberculosis* LAM family in Bulgaria based on the use of different molecular markers. A total of 101 previously spoligotyped *M. tuberculosis* strains were studied by LAM-specific PCR assay to detect an insertion of IS6110 in the specific genome region. On the whole, clear-cut results were obtained for most strains; spoligotype-based family was reassigned in some of them. At the same time, double bands were amplified in some cases and warrant further validation studies of this method. The higher MDR rate among LAM versus other genotype isolates was observed ($P = 0.04$). In conclusion, these results suggest a low (<4%) prevalence rate of LAM in Bulgaria (that is similar to its Balkan neighbors) and highlight the importance of using robust markers for correct detection of the LAM family.

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Introduction

Tuberculosis (TB) remains an important public health issue in Bulgaria. Although the number of new cases has declined since 2006 (41/100,000), the TB incidence rate in Bulgaria is still sufficiently high (26.7/100,000 in 2013), as well as multidrug-resistant TB (MDR-TB) rates among new TB cases (5.8%). Clinical and epidemiological characterization of the

major *Mycobacterium tuberculosis* (*M. tuberculosis*) subpopulations circulating in different geographic regions is an essential step for developing better diagnostics, therapeutics, and vaccines. The integration of molecular analysis of *M. tuberculosis* with an epidemiological analysis of clinical information provides a new tool to assess possible associations between *M. tuberculosis* strain types and the clinical and epidemiological characteristics of the disease [1].

* Corresponding authors at: The Stephan Angeloff Institute of Microbiology, 26 Acad. G. Bonchev Street, Sofia 1113, Bulgaria (V. Valcheva). St. Petersburg Pasteur Institute, 14 Mira Street, 197101 St. Petersburg, Russia (I. Mokrousov).

E-mail addresses: violeta_valcheva@mail.bg (V. Valcheva), imokrousov@mail.ru (I. Mokrousov).

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The population structure of *M. tuberculosis* is clonal and certain genetic families of this species have attracted more attention due to their global dissemination and/or pathogenic properties. A number of studies demonstrated an association with drug resistance of the Latin-American-Mediterranean (LAM) family [2–4]. On the other hand, other studies have shown high transmissibility of this strain, but not a significant association with increased virulence [5,6]. LAM was initially suggested by analysis of the global spoligotyping dataset [7] and its spoligotyping signature is absence of signals 21–24 and 33–36 (Fig. 1). However, abridged spoligoprofiles with long blocks of deleted spacers (e.g., SIT125) or homoplasmy spoligoprofiles (e.g., SIT803) make identification of such isolates uncertain [8–10]. To solve a phylogenetic ambiguity, the use of other molecular markers/methods may be helpful, in particular, MIRU-VNTR cluster analysis using reference LAM isolates (e.g., using MIRU-VNTRplus.org) or use of phylogenetically robust SNPs [11,12]. An IS6110 insertion in the specific genome region between genes *lpqQ* and *Rv0836c* (coding for hypothetical proteins) has also been suggested for detection of LAM [13,14] (Fig. 2) and evaluated in the present study.

Correct detection of the LAM family of *M. tuberculosis* has both phylogenetic and practical interest. The aim of this study was to evaluate the prevalence of LAM among *M. tuberculosis* strains circulating in Bulgaria based on the use of different molecular markers.

Materials and Methods

A study sample included 101 *M. tuberculosis* isolates obtained from different regions of the country and previously characterized by spoligotyping and other methods [15]. The DNA of the studied strains was extracted from 4 to 6 weeks Löwenstein-Jensen medium culture using the recommended method [16]. Spoligotyping was performed as described previously [17]. The individual spoligotyping patterns were compared with the international database SITVIT2 (Institut Pasteur de Guadeloupe) that is an updated version of the published SITVIT_WEB database [18].

Analysis of the IS6110 element specific for the LAM genetic family was performed as described previously [14]. In brief, a 205-bp band indicates a LAM strain due to the presence of an IS6110 element in a specific site in the genome, whereas a 141-bp band indicates a non-LAM strain lacking the IS6110 element in this site (Fig. 3).

To minimize the risk of laboratory cross-contamination during PCR amplification, each procedure (preparation of the PCR mixes, the addition of the DNA, the PCR amplification, and the electrophoretic fractionation) was conducted

in physically separated rooms. Negative controls (water) were included to control for reagent contamination.

A 2×2 χ^2 test was used to detect any significant difference between the two groups. Yates corrected χ^2 and P-values were calculated with 95% confidence interval at http://www.medcalc.org/calc/odds_ratio.php online resource.

Results and discussion

Application of the published rules for the definition of the major spoligotype clades and comparison with SITVIT2 global database permitted the assignment of most of the strains to the known spoligotype families. At the same time, some types were assigned to the convergent family (e.g., LAM/S) and use of further methods would be required. The PCR approach was used to define the LAM family [14] and all strains were tested for the presence or absence of the LAM-specific IS6110 insertion (Fig. 3; Table S1). Comparison of results obtained by different methods is shown in Table S1 where the spoligoprofiles are sorted for easier visualization of data. This permitted the identification of 3 differently sized groups of strains. The first group included 11 strains with 2 amplified bands. The multiplex PCR targeted an insertion of IS6110 in the specific genome region and was designed in such a way that either one of the other bands are amplified in case of the presence or absence of such insertion. Hence, these 11 strains present an apparent discrepancy. A double band is *a priori* impossible since this genome target appears to be unique. Only one of these strains was assigned to LAM in SITVIT_WEB (strain 54, SIT1280, although it was labeled as LAM/S in the previous version SpolDB4). Other strains with a double band belonged to T, X, Haarlem and unknown families; neither had a LAM-specific deletion block in the spoligoprofile (deleted signals 21–24). Furthermore, in 4 strains both bands were equally strong, thus excluding a possibility of contamination. 24-loci VNTR typing presented an additional proof of the lack of technical error (contamination): no strains had multiple alleles (not shown). An amplification of the additional band may be specific but not related to the targeted genome region. Further study of these cases is warranted.

The second group included 86 strains with a single amplified band, specific for the absence of the IS6110 insertion in the target locus, hence indicative of other than the LAM family (Table S1). Few observations can be made regarding the discrepancies with spoligotype-defined families. Four of the studied strains belonged to SIT41 defined in SITVIT_WEB as LAM7-TUR. However, no LAM-specific band was amplified in these strains; hence they cannot be defined as LAM. Indeed, another online resource for *M. tuberculosis* molecular typing

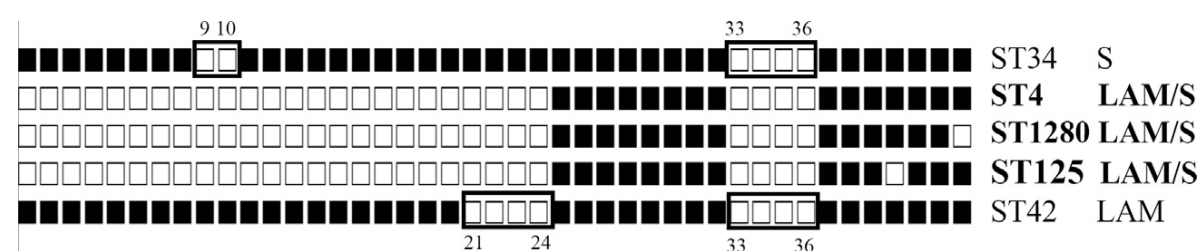


Fig. 1 – Visual presentation of the decision rules for definition of the LAM and S spoligotype families and ambiguous spoligotypes.

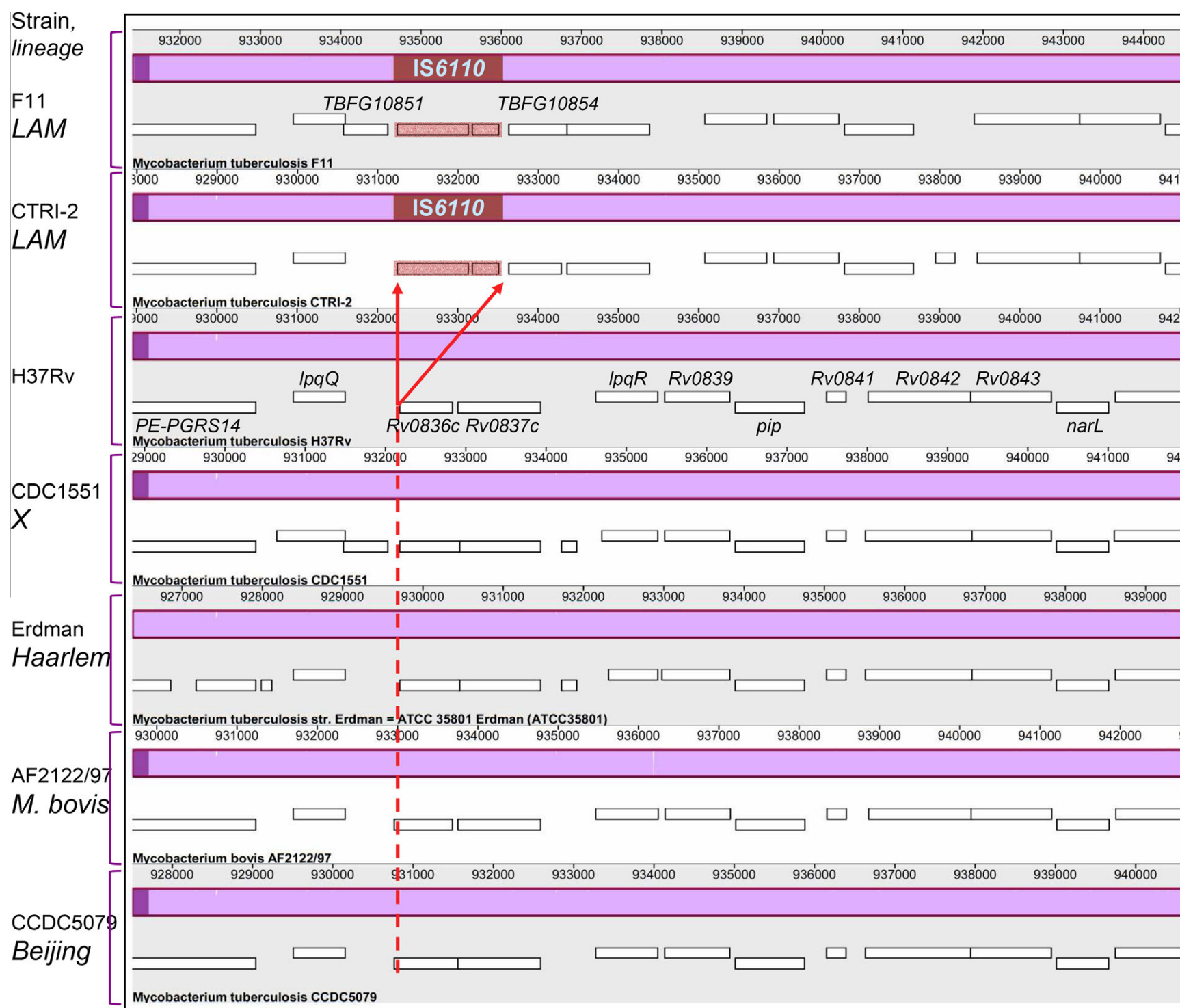


Fig. 2 – Specific IS6110 insertion characteristic of the LAM family. Complete genomes of *M. tuberculosis* strains were retrieved from GenBank and aligned using Mauve 2.3.1 program (<http://darlinglab.org/mauve/mauve.html>).

(MIRU-VNTRplus.org) defines the family of these strains as TUR, based on the analysis of different molecular markers, including VNTRs and SNPs. It is also interesting to note that all studied strains of the SIT125, which had an uncertain family definition (LAM/S in earlier SpolDB4 and T2/LAM3 in SITVIT_WEB), did not have a LAM-specific band; hence their family status cannot be LAM. Finally, two strains of SIT1588 had a single non-LAM band which is intriguing, since these strains had a prototype LAM signature (see SIT42 in Fig. 1), in addition to the deleted first signals. This profile may be a result of the convergent evolution of the DR locus, and more strains of this spoligotype SIT1588 should be studied in order to verify its family status.

Regarding the third and smallest group of strains, it included 4 strains with only LAM-specific band amplified. One of them—strain 52—was assigned to LAM in SITVIT_WEB; strain 8 was assigned to T5-RUS1, but it was recently shown to belong to LAM [9]. In its turn, strain 126 ('new' spoligotype, 'unknown' family in SITVIT_WEB) may indeed belong to LAM as confirmed herein by LAM-PCR. At the same time, strain 81 (SIT453/T) had LAM-specific band amplified, but intact

spacers 21–24; this discrepancy is unexpected and questions the value of this IS6110-PCR as LAM-specific.

The importance of the correct detection of LAM is explained by the fact that the heterogeneous genetic family of *M. tuberculosis*, LAM, has been shown in geographically distant settings to demonstrate remarkable pathogenic features. First, in Brazil, RD-Rio sublineage of LAM accounts for 37% of the total TB burden and was shown to be associated with pulmonary cavitation. Since cavitory TB is associated with a higher sputum-bacillary load, this finding supports the hypothesis that RD-Rio *M. tuberculosis* is associated with a more "severe" disease as a strategy to increase transmission, at least in some ethnic groups [5]. Secondly, LAM-RUS sublineage in central Russia (along with the Beijing genotype) was shown to be associated with MDR and clustering: the level of drug resistance in new cases was almost twice as high as the estimated average national level [2]. In the present study, 2 of the 4 isolates with a single LAM-specific PCR fragment were MDR, while two others were susceptible. Regarding 97 non-LAM isolates, 71 of them were susceptible and 10 were MDR. However, the higher MDR rate among LAM versus other

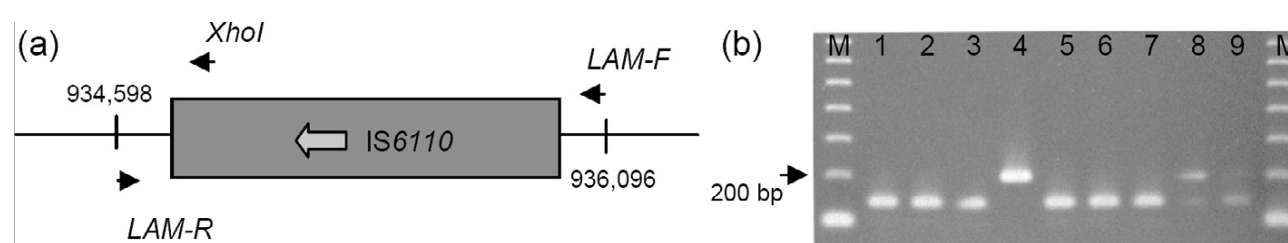


Fig. 3 – LAM-specific PCR: (a) schematic view of the genome region harboring LAM-specific IS6110 insertion in complete genome of strain F11. Three primers (arrows) are used in one reaction: LAM-F, LAM-R, XhoI. Non-LAM strain (without this IS6110-insertion): primers LAM-R and LAM-F amplify 141 bp fragment. LAM strain (with this IS6110 insertion): primers LAM-R and XhoI amplify 205 bp fragment (b) gel-electrophoresis. Lane 4 – LAM strain; lanes 8–9: mixed profile. M, molecular weights marker “100 bp DNA ladder” (GE Healthcare).

strains (2/4 versus 10/97; $P = 0.04$) should be regarded with caution due to the very small sample size of the LAM group, and further study is warranted.

Geographically, the 4 confirmed LAM isolates were not evenly dispersed across Bulgaria, but rather represented northern (Vratsa, Veliko Tarnovo and Pleven) and western (Sofia) parts of the country. Further placing these results in the wider regional context permitted this study to reveal an intriguing gradient of the LAM prevalence. Data on some countries are absent or scarce and estimations based only on spoligotypes are uncertain. These estimations were obtained keeping in mind that: (i) SIT4 cannot be assigned to any family based on spoligotyping alone; and (ii) LAM-TUR and LAM-CAM do not belong to LAM, but rather represent separate families within the Euro-American lineage. It appears that similar to Bulgaria (4%), the LAM rate is low/negligible in other Balkan countries: Turkey – 3.4%; Greece – 1–4%; Romania – 0% (SITVIT_WEB); and Albania – 0% [19]. In contrast, the LAM rate is higher in the northeastern neighbors of Bulgaria beyond the Balkans, reaching 17% in Southern Ukraine [20], 25%–39% in European Russia, and 42% in Belarus [9]. In its turn, this situation correlates with the prevalence of the rare Bulgarian LAM isolates mainly in the northern part of the country.

In conclusion, application of the LAM-specific PCR revealed a low (3%–4% depending on the marker used) prevalence rate of LAM family in the *M. tuberculosis* population in this country. Compared with spoligotyping, a phylogenetic family of 38 strains was revised or questioned: 27 strains were shown not to belong to LAM, while 11 more strains showed apparently discrepant results. This situation questions the global utility of such PCR and highlights the importance of using multiple markers for the molecular detection of the LAM family.

Ethical approval

Not required.

Funding

None.

Conflict of interest

N.R. and I.M. are Editorial Board members of the International Journal of Mycobacteriology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijmyco.2015.04.003>.

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