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Research paper

Insights on the *Mycobacterium tuberculosis* population structure associated with migrants from Portuguese-speaking countries over a three-year period in Greater Lisbon, Portugal: Implications at the public health level

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ABSTRACT

Tuberculosis among foreign-born patients is a key indicator of country-level epidemiological profiles and, of an increasing concern in Europe given the more intensified migratory waves of refugees. Since Portugal presents a lower immigrant-associated TB incidence rate when compared to other European countries, we sought to characterize the epidemiology and transmission dynamics among the foreign-born population coming from Portuguese-speaking countries that are associated with higher TB incidences. In the present study we analyzed 133 *Mycobacterium tuberculosis* isolates obtained from foreign-born individuals over a three-year period in Lisbon, Portugal, using molecular epidemiological methods such as spoligotyping and 24-loci MIRU-VNTR. Moreover, all strains were subjected to drug susceptibility testing. The genetic profiles obtained suggest that strain importation from Portuguese speaking countries plays a less important role in TB epidemiology but instead argue in favor of a high degree of penetrance of Portuguese endemic strains to the migrant population, including multidrug resistant strains, which is particularly relevant to active screening programs.

1. Introduction

Historically, tuberculosis (TB) among migrants or foreign-born patients has been a cause of public health concern and key indicator of country-level TB epidemiological profiles (Europe, 2017). In Europe, a renewed attention has been given to this topic due to refugee-associated mass migratory movements from North Africa and Central Asia towards European countries (Tavares et al., 2017; van der Werf et al., 2017).

Unlike countries such as the United Kingdom, Sweden or Norway, Portugal has a lower immigrant-associated TB notification rate (15.9% in 2015) that is traditionally driven by migratory movements originating from Portuguese-speaking countries, most of which located on sub-Saharan Africa (Europe, 2017). Despite this, the foreign-born population is regarded by Portuguese Health Authorities as a special risk group in Portugal (Direcção Geral de Saúde, 2014).

Among migrants, a different aspect pertains the mode of TB transmission: i) TB may have been acquired in the home country, during a return travel or on the way to the host country; ii) be the result of a latent TB infection (LTBI) acquired prior to arrival in the country; or iii), is the result of a new infection acquired in the host country (Tavares et al., 2017; Zammarchi et al., 2015). In Portugal, the mean time for the onset of active TB in these patients occurs 2 years after arrival in the country, conveying the notion that rather than carrying TB at the time of arrival, a significant proportion of these patients may have been infected after entering the country and, may not play a significant role towards importation of *Mycobacterium tuberculosis* strains that are not

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endemic to the country (Direcção Geral de Saúde, 2014). However, this notion has not been previously confirmed by molecular data nor did the *M. tuberculosis* population structure been adequately characterized across different immigrant sub-populations. Hence, the present investigation was meant to clarify if TB associated with foreign-born patients in Portugal is mainly driven by *M. tuberculosis* strain importation from the patients' country of origin or, if it is driven by local endemic strains undergoing clonal expansion at the community-level. To address this issue, we characterized a set of *M. tuberculosis* clinical strains isolated from patients born in Portuguese-Speaking African countries between 2008 and 2010. Over this period, the prevalence of TB in foreign-born citizens increased from 13.7% to 16.2% and, according to the last nationwide populational survey conducted in the country, in 2011, the foreign population residing in the country amounted to 394,496 individuals which comprised a 70% increase along the previous decade (Instituto Nacional de Estatística, 2012). From these, 92,617 (23.5%) individuals were born in Portuguese-Speaking African countries. Recent data on TB notifications among immigrants by country of origin is lacking but, data from 2009 shows that at least 62.6% of all TB cases diagnosed among foreign-born patients were from patients born in Portuguese-speaking African countries (Direcção Geral de Saúde, 2010). Also in 2011, and according with WHO data, the TB incidence in these countries ranged between 94 and 548 cases per 100,000 habitants in Sao Tome and Principe and, Mozambique, respectively, therefore posing a potential source for new cases in Portugal that warrants further investigation (World Health Organization, 2013).

2. Methods

2.1. Clinical isolates

A total of 133 clinical isolates, each corresponding to a different foreign-born patient from a Portuguese-Speaking African country, recovered over a three-year period (2008–2010) at the Laboratory of Public Health of Regional Administration of Lisbon were made available for strain typing. This sample of 133 (14.6%) isolates corresponds to a sub-set of a total of 908 *M. tuberculosis* isolates recovered over the same period and for which it was possible to retrieve the patient's country of origin. The Lisbon region concentrates the majority (51.6%) of foreign-born residents in the country and the prevalence of foreign-born associated TB cases in this study population is comparable to nation-wide aggregated data for the same period (Europe, 2017).

2.2. Drug susceptibility testing

All isolates were tested for first- and second-line drug susceptibility by the BACTEC™ MGIT™ 960 system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) using standardized procedures (Siddiqi and Rüsch-Gerdes, 2006).

2.3. Genotyping and mutational analysis of resistance associated genes

Genotyping was initially carried out by Spoligotyping as previously described (Kamerbeek et al., 1997). Briefly, a single-tube multiplex PCR amplification of 43 spacer regions of the direct repeat (DR) locus was done using oligonucleotide pair DRa (5'-Biotin-GGTTTTGGGTCTGAC GAC-3′) and DRb (5'-CCGAGAGGGGACGAAAC-3′) and 20 ng of genomic DNA. An in-house produced membrane with amino-linked immobilized probes was used in a reverse hybridization protocol, as described previously, using the amplicons obtained by the previous multiplex PCR step (Kamerbeek et al., 1997). Spoligotyping profiles were assigned to lineage, clade and shared international type (SIT) using the rules described in SITVITWEB and SITVIT2 international databases (Couvin et al., 2018; Couvin and Rastogi, 2015; Demay et al., 2012).

Twenty-four-*loci* Mycobacterial Interspersed Repetitive Unit – Variable Number of Tandem Repeat (MIRU-VNTR) was carried out using a multiplex amplification procedure followed by amplicon size determination by capillary electrophoresis, as described previously (Supply, 2005; Supply et al., 2006). Single-locus amplification was performed for all loci that did not amplify initially and subsequent determination of amplicon size was done by agarose gel electrophoresis (Perdigao et al., 2018).

RD-based typing was conducted for RD^{RIO} for all isolates assigned to a Latin American and Mediterranean (LAM) clade using a multiplex PCR-based method (Gibson et al., 2008).

Drug resistance associated mutations were characterized by PCR amplification of known resistance associated genes and Sanger sequencing analysis as previously described (Perdigao et al., 2014).

2.4. Statistical analysis

Statistical analyses were carried out using the IBM© SPSS© Statistics v.21 (IBM Corporation, Armonk, NY, USA) or R (R Foundation for Statistical Computing, Vienna, Austria). SIT or Clade prevalence across different datasets were compared using Fisher's exact test under a two-sided probabilistic distribution.

3. Results

3.1. TB among immigrants from Portuguese-speaking African countries and M. tuberculosis population structure

From a total of 133 clinical isolates, each corresponding to a foreign-born patient originating from a Portuguese-speaking country, three countries were highly represented by comprising a total of 114 of the 133 patients: Angola ($n = 51$), Cape Verde ($n = 33$) and Guinea-Bissau ($n = 30$). Further demographic indicators are summarized in Supplementary Table S1. Moreover, 9/133 (6.8%) isolates presented resistance to one or more first-line drugs, of which four were classed as multidrug resistant (MDR; i.e. concomitant resistance to isoniazid and rifampicin). No extensively drug resistant (XDR; MDR with cumulative resistance to a fluoroquinolone and a second-line injectable drug) isolate was detected although two of the four MDR-TB isolates presented resistance to ofloxacin and capreomycin, respectively (pre-XDR-TB).

The overall population structure unveiled by Spoligotyping indicated a high prevalence of LAM strains ($n = 77$; 57.9%) followed by the "ill-defined" T strains ($n = 15$; 11.3%) and Beijing strains ($n = 12$; 9.0%). The prevalence of the latter is noteworthy as it may imply introduction of these unusual strains in Portugal. The most prevalent SITs were: SIT20/LAM1 ($n = 28$; 21.169%) and SIT42 ($n = 18$; 13.5%). As the number of cases other than those from the sub-sets originating from the three most represented countries were too low to undertake a robust population structure comparison, we instead compared the population structure of the immigrant sub-populations from Angola, Cape Verde and Guinea-Bissau. All subsets exhibited similar structures with Cape Verde and Guinea-Bissau subsets diverging from the *M. tuberculosis* population structure known to exist at the respective geographical origin (Fig. 1) (Demay et al., 2012). For Angolan immigrants, as the populations structures for Angola and Portugal are extremely similar, there were no clear differences in this comparison (Demay et al., 2012; Perdigao et al., 2018). In fact, the core of the population sub-structures among immigrants appeared to converge with the overall population structure available in SITVIT for Portugal ($n = 782$ strains, including this study) and is suggestive of a high degree of penetrance of Portuguese circulating strains into the migrant population (Fig. 1; Table 1) (Demay et al., 2012).

The above hypothesis was further supported by comparative statistical analyses demonstrating that the prevalence of most spoligotyping lineages and respective clades across each populational sub-set did not significantly differ from the overall *M. tuberculosis* population

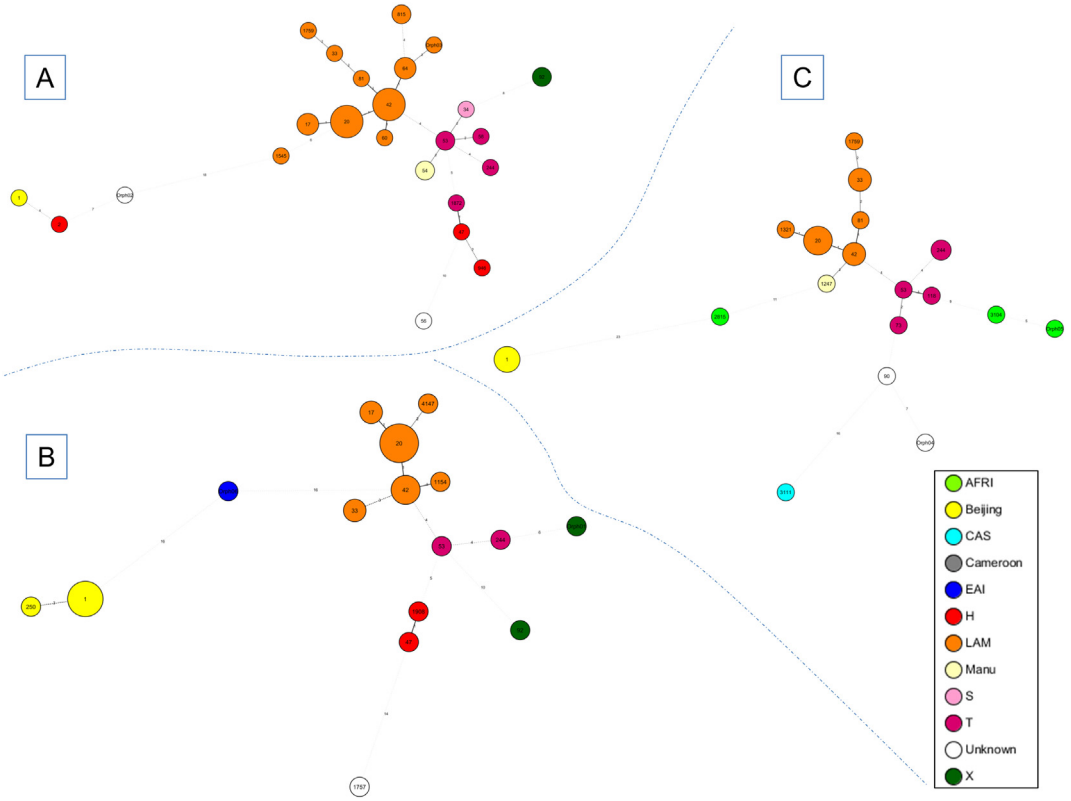

Fig. 1. Minimum Spanning Trees (MSTs) drawn using MLVA Compare software (Genoscreen, Lille, France and Ridom Bioinformatics, Münster, Germany). These trees were based on all available spoligotyping patterns in this study, focusing on: (A) Angola (n = 51 isolates), (B) Cape Verde (n = 33 isolates) and (C) Guinea-Bissau (n = 30 isolates). Nodes were colored in function of lineages, and their sizes were proportional to the number of isolates for a given SIT. Thickness and/or shape of branches (continuous, dashed, dotted, black or gray) varied in function of spacer changes between spoligotyping patterns. The number of changes is indicated on branches. A legend is displayed in bottom right of figure.

structure in Portugal. However, among the three most well represented immigrant subpopulations (Angola, Cape Verde and Guinea-Bissau), unusually prevalent strains were detected: i) Beijing strains among Cape Verde and Guinea-Bissau immigrants; ii) AFRI strains among Guinea-Bissau immigrants; and iii) LAM strains among immigrants from Angola (Table 2). Similarly, the SIT-specific compositional distribution showed an unusual high prevalence of SIT1/Beijing isolates among immigrants from Cape Verde and Guinea-Bissau and an increased prevalence of SIT42/LAM9 strains in immigrants from Angola (Table 3). Furthermore, SIT33/LAM3 strains were also detected among Guinea-Bissau born patients at a high relative prevalence when compared to SIT33/LAM3 isolates in Portugal. These observations also lend support to the emergence of specific strains that are present at an increased frequency at the respective countries of origin driven by immigration.

3.2. Distribution of RD^{RIO} strains across SITs/clades and subpopulations

Given the high predominance of LAM strains across all sample subsets and, in an attempt to gain further insight on the population structure of this prevalent clade using an additional macro-epidemiological marker, we sub-typed all LAM strains from patients born in Angola, Cape Verde and Guinea-Bissau (n = 65) based on RD^{RIO}. Among 58 isolates for which amplification did occur, 47 (81.0%) belonged to the RD^{RIO} sub-lineage. Wild-type strains for this RD (n = 11; 19.0%) were found across all three subsets and were mainly associated with SIT33/LAM3, SIT64/LAM6 and SIT1759/LAM3 (Table 4). The three latter SITs were also detected in Portugal at frequencies comparable to those found in the studied foreign-born patients except for the above mentioned SIT33/LAM3 strains in Guinea-Bissau (Table 3). These findings argue in favor of a common LAM substructure among foreign-born patients from Angola, Cape Verde and Guinea-Bissau diagnosed in Portugal over the study period.

3.3. Genetic clusters associated with drug resistance and molecular basis of resistance

Next, to investigate the emergence and transmission dynamics concerning drug resistant isolates among these sub-populations of foreign-born patients, the nine drug resistant clinical isolates (Angola, n = 5; Cape Verde, n = 1; Mozambique, n = 2; Guinea-Bissau, n = 1) were further genotyped by 24-loci MIRU-VNTR enabling the identification of three genetic clusters (herein defined as a group of more than one isolate sharing undistinguishable MIRU-VNTR profiles; Fig. 2). This approach enabled the comparison with a much vaster dataset currently available at the CPLP-TB database (http://cplp-tb.ff.ulisboa.pt). Two of the identified clusters corresponded to the endemic Lisboa3-A and Lisboa3-B clusters, highly associated with MDR and XDR-TB, respectively, since the 90's in Portugal (Perdigao et al., 2014). The molecular basis of resistance herein determined for the drug resistant isolates is completely congruent with the molecular resistance signature of the Lisboa3 clade (Fig. 2) (Perdigao et al., 2014). Briefly, all isoniazid-resistant isolates (n = 4) were associated with either Lisboa3-A or Lisboa3-B genetic clusters as defined by 24-loci MIRU-VNTR, with the molecular basis of resistance driven by *inhA* promoter mutations (C-15 T) albeit one isolate bore a concomitant frameshift mutation at *katG* (968delA). Similarly, all Lisboa3 MDR isolates showed the *rpoB* S531 L (n = 4) mutation underpinning rifampicin resistance. One non-clustered SIT92/X3 isolate displaying rifampicin mono-resistance bore a H526D mutation in *rpoB*. Streptomycin was mainly associated with *rpsL* mutations (n = 7) with one isolate also showing a *rrs* (A807C) mutation while the only pyrazinamide-resistant isolate bore a 10 bp insertion in *pncA* gene. Also, the only ethambutol-resistant isolate did not exhibit

Table 1
Predominant SITs and global distribution according to SITVITEXTEND.

SIT/Lineage	No. in study (%)	% in study vs. database	No. in Portugal (%)[a]	Global Distribution in Countries with ≥ 2% of a given SIT[b]	Global Distribution in Regions with ≥ 2% of a given SIT[c]
1/Beijing	11 (8.27)	0.1	18 (2.77)	CN = 19.05, US = 18.82, JP = 10.82, ZA = 7.78, RU = 6.57, VN = 3.66, IN = 3.6, PE = 2.95, MY = 2.78	Eastern Asia = 32.08, North America = 18.86, Southeastern Asia = 10.02, Southern Africa = 7.78, Northern Asia = 6.57, Southern Asia = 5.96, Northern Europe = 3.43, South America = 3.4, Western Asia = 2.8, Western Europe = 2.41
17/LAM2	5 (3.76)	0.63	8 (1.23)	BR = 33.33, VE = 22.69, US = 15.08, HT = 6.97, ES = 3.68, GP = 2.53, CO = 2.28	South America = 61.22, North America = 15.08, Caribbean = 11.28, Southern Europe = 5.58
20/LAM1	29 (21.8)	2.67	117 (18.03)	BR = 19.03, US = 14.61, PT = 13.42, HT = 6.25, NA = 5.7, FR = 4.5, VE = 3.86, ZA = 2.48, ES = 2.3, AR = 2.02	South America = 30.79, Southern Europe = 16.18, North America = 14.61, Western Europe = 8.46, Southern Africa = 8.18, Caribbean = 7.81, East Africa = 3.4, Central Africa = 2.67, Northern Europe = 2.39, West Africa = 2.3
33/LAM3	6 (4.51)	0.4	9 (1.39)	ZA = 21.83, PE = 14.1, BR = 12.22, US = 10.68, AR = 10.41, ES = 5.98, CL = 3.69, FR = 3.63, IT = 3.09, HN = 2.89	South America = 42.11, Southern Africa = 21.83, North America = 10.68, Southern Europe = 10.07, Western Europe = 5.51, Central America = 3.63, North Africa = 2.15
42/LAM9	18 (13.53)	0.47	57 (8.72)	BR = 15.26, US = 10.32, CO = 7.44, MA = 6.14, IT = 5.69, FR = 4.42, AR = 3.43, PE = 3.22, ES = 2.91, VE = 2.89, ZA = 2.76, RU = 2.21, HT = 2.11	South America = 35.28, Southern Europe = 10.66, North America = 10.32, Western Europe = 8.24, North Africa = 7.46, Northern Europe = 4.26, Caribbean = 3.69, East Africa = 3.38, Central America = 3.09, Southern Africa = 2.76, Northern Asia = 2.21
47/H1	3 (2.26)	0.17	22 (3.39)	US = 13.81, BR = 7.72, IT = 7.49, AT = 7.31, PE = 6.85, FR = 6.04, FI = 5.46, CZ = 3.42, ES = 3.25, SE = 3.08, AR = 2.61, MA = 2.55, NL = 2.26, CF = 2.15, TR = 2.03	South America = 19.15, Western Europe = 18.46, North America = 14.1, Southern Europe = 12.59, Northern Europe = 9.52, Eastern Europe = 6.56, Western Asia = 3.66, North Africa = 3.31, Caribbean = 2.5, Central Africa = 2.38
53/T1	5 (3.76)	0.07	41 (6.32)	US = 11.79, FR = 7.04, BR = 6.63, IT = 4.75, TR = 4.44, ZA = 4.33, PE = 4.23, AT = 3.05, CN = 2.78, FI = 2.61, MX = 2.54, ET = 2.31, SA = 2.23, AR = 2.16, ES = 2.06	South America = 17.68, Western Europe = 13.95, North America = 12.02, Southern Europe = 8.66, Western Asia = 7.86, Northern Europe = 6.67, East Africa = 4.61, Southern Africa = 4.43, Eastern Asia = 3.8, North Africa = 3.14, Eastern Europe = 2.91, Central America = 2.88, Caribbean = 2.84, Southern Asia = 2.82
54/Manu2	2 (1.5)	0.7	2 (0.31)	IN = 18.25, CN = 15.79, EG = 11.93, SA = 7.02, US = 7.02, ZA = 5.61, IQ = 4.91, RU = 3.51, ET = 3.16, BR = 2.46	Southern Asia = 18.95, Eastern Asia = 15.79, Western Asia = 14.74, North Africa = 12.63, North America = 7.02, Southern Africa = 5.61, East Africa = 4.91, Northern Asia = 3.51, South America = 3.51, Northern Europe = 2.11, Southern Europe = 2.11, Central Asia = 2.11
64/LAM6	3 (2.26)	0.63	17 (2.62)	BR = 44.21, US = 19.79, GF = 4.84, PT = 4.21, PE = 2.53, FR = 2.53, AR = 2.32	South America = 57.68, North America = 19.79, Southern Europe = 5.05, Western Europe = 5.05, Western Asia = 2.53
73/T	2 (1.5)	0.61	4 (0.62)	BR = 20.67, IT = 11.25, US = 10.94, ZA = 8.51, FR = 6.08, AR = 4.26, CN = 3.65, MZ = 3.04, MX = 2.43, GF = 2.13	South America = 28.88, Southern Europe = 14.29, Western Europe = 10.94, North America = 10.94, Southern Africa = 8.51, East Africa = 6.08, Eastern Asia = 3.65, Central America = 3.04, West Africa = 2.13, Western Asia = 2.13
81/LAM9	3 (2.26)	7.14	1 (0.15)	CU = 38.1, ES = 14.29, BR = 9.52, PT = 9.52, BE = 7.14, IT = 4.76, MX = 4.76, CL = 2.38, ZW = 2.38, ZA = 2.38, AR = 2.38, FR = 2.38	Caribbean = 38.1, Southern Europe = 28.57, South America = 14.29, Western Europe = 9.52, Central America = 4.76, East Africa = 2.38, Southern Africa = 2.38
92/X3	3 (2.26)	0.64	1 (0.15)	ZA = 43.99, US = 18.03, BR = 11.37, MX = 3.86, GB = 3.0, PE = 2.36	Southern Africa = 43.99, North America = 18.03, South America = 15.88, Central America = 4.94, Northern Europe = 4.51, East Africa = 2.79, Western Europe = 2.58, Southern Europe = 2.15
244/T1	4 (3.01)	2.56	18 (2.77)	BR = 22.44, PT = 14.1, ZA = 12.18, BD = 8.97, FR = 8.33, ZM = 5.13, GW = 5.13, AO = 3.85, TZ = 3.85, US = 3.21	South America = 23.72, Southern Europe = 15.38, East Africa = 12.82, Southern Africa = 12.18, Southern Asia = 10.26, Western Europe = 9.62, West Africa = 5.77, Central Africa = 3.85, North America = 3.21
815/LAM11-ZWE	2 (1.5)	1.32	0 (0.00)	ZM = 41.72, ZA = 19.21, BE = 17.88, ZW = 5.96, MZ = 5.96, TZ = 2.65	East Africa = 57.62, Southern Africa = 19.21, Western Europe = 17.88
1752/LAM1	2 (1.5)	28.57	4 (0.62)	PT = 85.71, US = 14.29	Southern Europe = 85.71, North America = 14.29
1759/LAM3	2 (1.5)	40.0	3 (0.46)	PT = 100.0	Southern Europe = 100.0

[a] Number and percentage of strains isolated in Portugal according to SITVITEXTEND (excluding this study), i.e. n = 782–133 = 649 isolates.
[b] The 2 letter country codes are according to http://en.wikipedia.org/wiki/ISO_3166-1_alpha-2; countrywide distribution is only shown for SITs with ≥2% of a given SIT as compared to their total number in the SITVIT database.
[c] Worldwide distribution is reported for regions with > 2% of a given SIT as compared to their total number in the SITVIT database.

Table 2
Prevalence of different spoligotyping lineages and clades across the most well represented datasets: Angola, Cape Verde and Guinea-Bissau and comparison with the overall population structure available on SITVITEXTEND. Only lineages and clades represented by more than one isolate are shown.

Spoligotyping Lineage/Clade	No. Isolates by Country of Origin (%)						SITVITEXTEND				
	Angola	p^a	Cape Verde	p^a	Guinea-Bissau	p^a	Portugal	Angola	p^b	Guinea-Bissau	p^b
Total	51(100)		33(100)		30(100)		782 (100)	89(100)		436(100)	
AFRI	0(0)	1.000	0(0)	1.000	3(10)	**0.001**	3(0.4)	0(0)	1.000	196(45)	< **0.001**
AFRI-1	0(0)	1.000	0(0)	1.000	2(6.7)	**0.012**	3(0.4)	0(0)	1.000	187(42.9)	< **0.001**
Beijing	1(2)	1.000	7(21.2)	< **0.001**	4(13.3)	**0.032**	30(3.8)	0(0)	0.364	15(3.4)	**0.027**
Haarlem/H	3(5.9)	0.790	2(6.1)	1.000	0(0)	0.159	61(7.8)	1(1.1)	0.137	37(8.5)	0.155
H1	1(2)	1.000	1(3)	1.000	0(0)	0.620	29(3.7)	0(0)	0.364	22(5)	0.385
H3	1(2)	1.000	1(3)	1.000	0(0)	0.622	30(3.8)	1(1.1)	1.000	15(3.4)	0.613
LAM	35(68.6)	**0.029**	18(54.5)	0.860	14(46.7)	0.579	411(52.6)	51(57.3)	0.210	77(17.7)	< **0.001**
LAM1	13(25.5)	0.607	9(27.3)	0.526	5(16.7)	0.654	176(22.5)	17(19.1)	0.398	11(2.5)	**0.002**
LAM2	3(5.9)	0.118	2(6.1)	0.177	0(0)	1.000	17(2.2)	2(2.2)	0.354	2(0.5)	1.000
LAM3	2(3.9)	1.000	2(6.1)	0.376	4(13.3)	**0.032**	30(3.8)	4(4.5)	1.000	1(0.2)	< **0.001**
LAM6	4(7.8)	0.105	0(0)	0.620	0(0)	0.619	26(3.3)	5(5.6)	0.724	0(0)	1.000
LAM9	10(19.6)	0.127	5(15.2)	0.587	4(13.3)	0.777	95(12.1)	17(19.1)	1.000	46(10.6)	0.549
LAM11-ZWE	2(3.9)	**0.047**	0(0)	1.000	0(0)	1.000	4(0.5)	0(0)	0.131	0(0)	1.000
MANU	2(3.9)	0.081	0(0)	1.000	1(3.3)	0.232	6(0.8)	0(0)	0.131	6(1.4)	0.374
MANU2	2(3.9)	0.081	0(0)	1.000	1(3.3)	0.232	6(0.8)	0(0)	0.131	4(0.9)	0.284
T	5(9.8)	0.242	2(6.1)	0.146	5(16.7)	1.000	130(16.6)	24(27)	**0.017**	8(1.8)	0.001
T1	4(7.8)	0.385	2(6.1)	0.418	4(13.3)	1.000	101(12.9)	21(23.6)	**0.022**	41(9.4)	0.517
T2	0(0)	1.000	0(0)	1.000	0(0)	1.000	7(0.9)	1(1.1)	1.000	2(0.5)	1.000
X	2(3.9)	0.372	2(6.1)	0.207	0(0)	1.000	19(2.4)	0(0)	0.131	4(0.9)	1.000
X2	0(0)	1.000	1(3)	0.220	0(0)	1.000	5(0.6)	0(0)	1.000	0(0)	1.000
X3	2(3.9)	0.164	1(3)	0.367	0(0)	1.000	10(1.3)	0(0)	0.131	2(0.5)	1.000
Total	51(100)		33(100)		30(100)		782 (100)	89(100)		436(100)	

[a] p value obtained from the comparison of the respective subset to the respective prevalence of each clade among the overall Portuguese Population available on SITVITEXTEND; statistically significant values (< 0.05) are highlighted in Bold.
[b] p value obtained from the comparison of the respective subset to the respective prevalence of each clade among the overall population available on SITVITEXTEND for the country of origin; statistically significant values (< 0.05) are highlighted in Bold.

Table 3
SIT prevalence across the most well represented datasets: Angola, Cape Verde and Guinea-Bissau and comparison with the overall population structure available on SITVITEXTEND. Only SITs represented by more than one isolate are shown.

Spoligotyping SIT/Clade	No. Isolates by Country of Origin (%)						SITVITEXTEND		
	Angola	p^a	Cape Verde	p^a	Guinea-Bissau	p^a	Portugal	Angola	Guinea-Bissau
Total	51(100)		33(100)		30(100)		782 (100)	89(100)	436(100)
SIT1/Beijing	1(2.0)	1.000	6(18.2)	**0.000**	4(13.3)	**0.007**	18 (2.77)	0 (0.00)	15 (3.44)
SIT17/LAM2	3(5.9)	**0.025**	2(6.1)	0.058	0(0.0)	1.000	8 (1.23)	0 (0.00)	0 (0.00)
SIT20/LAM1	12(23.5)	0.110	8(24.2)	0.144	5(16.7)	0.794	117 (18.03)	17 (19.10)	11 (2.52)
SIT33/LAM3	1(2.0)	0.470	2(6.1)	0.070	3(10.0)	**0.008**	9 (1.39)	1 (1.12)	0 (0.00)
SIT42/LAM9	9(17.6)	**0.015**	4(12.1)	0.301	3(10.0)	0.480	57 (8.72)	15 (16.85)	38 (8.72)
SIT47/H1	1(2.0)	1.000	1(3.0)	0.619	0(0.0)	1.000	22 (3.39)	0 (0.00)	18 (4.13)
SIT53/T1	2(3.9)	1.000	1(3.0)	1.000	1(3.3)	1.000	41 (6.32)	12 (13.48)	10 (2.29)
SIT54/Manu2	2(3.9)	**0.020**	0(0.0)	1.000	0(0.0)	1.000	2 (0.31)	0 (0.00)	2 (0.46)
SIT64/LAM6	3(5.9)	0.118	0(0.0)	1.000	0(0.0)	1.000	17 (2.62)	3 (3.37)	0 (0.00)
SIT81/LAM9	1(2.0)	0.119	0(0.0)	1.000	1(3.3)	0.073	1 (0.15)	0 (0.00)	0 (0.00)
SIT92/X3	2(3.9)	**0.011**	1(3.0)	0.079	0(0.0)	1.000	1 (0.15)	0 (0.00)	2 (0.46)
SIT244/T1	1(2.0)	1.000	1(3.0)	0.548	2(6.7)	0.166	18 (2.77)	6 (6.74)	8 (1.83)
SIT815/LAM11-ZWE	2(3.9)	**0.004**	0(0.0)	1.000	0(0.0)	1.000	0 (0.00)	0 (0.00)	0 (0.00)
SIT1759/LAM3	1(2.0)	0.224	0(0.0)	1.000	1(3.3)	0.140	3 (0.46)	0 (0.00)	0 (0.00)
Total	51(100)		33(100)		30(100)		782 (100)	89(100)	436(100)

[a] p value obtained from the comparison of the respective subset to the respective prevalence of each SIT among the overall Portuguese Population available on SITVITEXTEND; Statistically significant values (< 0.05) are highlighted in Bold.

any mutation at the classical *embB* ethambutol resistance determining region.

The third cluster (PT-03) comprised two clinical isolates recovered from two patients from Mozambique. Comparison with the CPLP-TB database shows that to this date PT-03 comprises four clinical isolates mono-resistant to streptomycin, two from Mozambique-born patients and the remaining two from Portuguese nationals (Perdigao et al., 2018). Similarly, the PT000301 isolate was found to belong to the PT-01 cluster comprising two streptomycin mono-resistant isolates, where the remaining isolate was isolated from a Portuguese-born individual. The remainder isolates (PT000351 and PT000363) failed to cluster with any isolate profile available in our database and were therefore classified as non-clustered.

4. Discussion

In the present study, we have characterized the population structure of *M. tuberculosis* among foreign-born patients originating from African

Table 4
Distribution of strains of the RD^{RIO} sublineage by patient country of origin and SIT/Clade across foreign-born patients from Angola, Cape Verde and Guinea-Bissau.

Country of Origin	RD^{RIO} Deletion/Sublineage		Wild-type RD^{RIO}	
	n (%)[a]	SITs/Clades (n)	n (%)[a]	SITs/Clades (n)
Angola	26 (83.9)	SIT17/LAM2(1); SIT20/LAM1 (11); SIT42/LAM9 (9); SIT60/LAM4 (1); SIT81/LAM9 (1); SIT815/LAM11-ZWE (2); SIT1545/LAM1 (1)	5 (16.1)	SIT33/LAM3 (1); SIT64/LAM6 (3); SIT1759/LAM3 (1)
Cape Verde	13 (86.6)	SIT17/LAM2(2); SIT20/LAM1 (11); SIT42/LAM9 (4); SIT1154/LAM9 (1); Orphan/LAM (1)	2 (13.3)	SIT33/LAM3 (2)
Guinea-Bissau	8 (66.6)	SIT20/LAM1 (3); SIT42/LAM9 (1); SIT81/LAM9 (1)	4 (33.3)	SIT33/LAM3 (3); SIT1759/LAM3 (1)
Total	47 (81.0)	SIT17/LAM2(1); SIT20/LAM1 (11); SIT42/LAM9 (9); SIT60/LAM4 (1); SIT81/LAM9 (1); SIT815/LAM11-ZWE (2); SIT1545/LAM1 (1)	11 (19.0)	SIT33/LAM3 (6); SIT64/LAM6 (3); SIT1759/LAM3 (2)

[a] Percentage values are expressed as a fraction of the total number of isolates with results for RD^{RIO} sublineage within each country of origin.

Portuguese-Speaking countries. The data we have obtained show a convergence to LAM spoligotypes but, regarding specific clades, the data shows divergence that may be associated with the importation of specific genotypes that are uncommon in Portugal. This is true for *Mycobacterium africanum* (AFRI) genotypes and Beijing isolates from Guinea-Bissau (Groenheit et al., 2011; Rabna et al., 2015). A higher prevalence of Beijing strains among Cape Verde immigrants was also noted, particularly when compared with the overall Portuguese population structure, however no available data on Cape Verde currently exists.

Across different continents, LAM strains appear to be the hallmark of Portuguese-speaking countries as recently shown and, potentially driven by back-to-Africa historical migrations from Europe (Brynildsrud et al., 2018; Perdigao et al., 2018). In Portugal, e.g., the high LAM prevalence contrasts with the overall LAM prevalence in Southern or Western Europe and this is even more notorious when concerning the LAM1 clade (Couvin et al., 2018; Demay et al., 2012). Herein, SIT20/LAM1 shows a comparable prevalence across the three foreign-born sub-populations studied and Portugal. A limitation to this comparison resides in the fact that the sample size for countries of origin other than Angola, Cape Verde and Guinea-Bissau are small and could not be compared to the overall population structure in Portugal and respective countries. This partially owes to the fact that TB cases among foreign born patients from Mozambique and, Sao Tome and Principe account for a lesser number of TB cases than the other three countries herein analyzed in greater detail. In fact, nationwide data from 2009 shows that 57.6% of all foreign-born TB patients were from Angola, Cape Verde and Guinea-Bissau with TB patients born in Mozambique accounting for 5.3% off all foreign-born cases in that year (n = 414). Furthermore, this study was focused on Lisbon Health region which, given its relatively small area (14,572 km^2), encompasses most of the foreign-born population coming from Portuguese-speaking African countries (Instituto Nacional de Estatística, 2012).

Regarding drug resistance across these patients, we detected four MDR-TB foreign-born cases, all associated with Lisboa3 clusters, along with five non-MDR isolates. The MDR-TB strains are highly likely to have been acquired in Portugal, but the other five non-MDR cases leave margin to speculate if: i) these strains were brought to Portugal from Mozambique and Angola, respectively, and have afterwards spread in the community to Portuguese-born individuals; or, ii) if TB in these foreign-born patients was acquired from strains that at the time were already circulating in Portugal. The existing data precludes us from reaching a conclusion although it is obvious the importance of gaining further control of TB transmission in the foreign-born population, particularly among those originating from Portuguese-speaking countries. Herein, and contrary to recent studies in other European countries where most TB cases among foreign-born patients or asylum seekers were non-clustered, all but two isolates (22.2%) were non-clustered (Andres et al., 2017).

Our data does support and corroborate TB transmission to migrant communities and, that TB among migrants may not be potentiated by the higher TB incidence in the country of origin but, by factors associated with economic deprivation and social marginalization (Tavares et al., 2017). These aspects also do lead to an increased risk for TB-HIV infection, also resulting from a limited access to healthcare (Matteelli et al., 2016; Tavares et al., 2017). Some derived implications at the public health level pertain the current strategies for TB detection in the immigrant population. While screening programs aimed at detecting and treating LTBI in immigrants appears to be cost-effective and lead to health improvement, doing so through IGRA-based screening and upon arrival to the country may not prove to be the most cost-effective strategy. In the present scenario, chest X-ray and standard early laboratory detection of *M. tuberculosis* along with more effective administrative procedures to allow prompt access to TB care for immigrants, could be the most cost-effective strategy for active TB detection coupled with targeted community-level screening as to circumvent cryptic transmission chains that would otherwise be missed by active case finding (Zammarchi et al., 2015).

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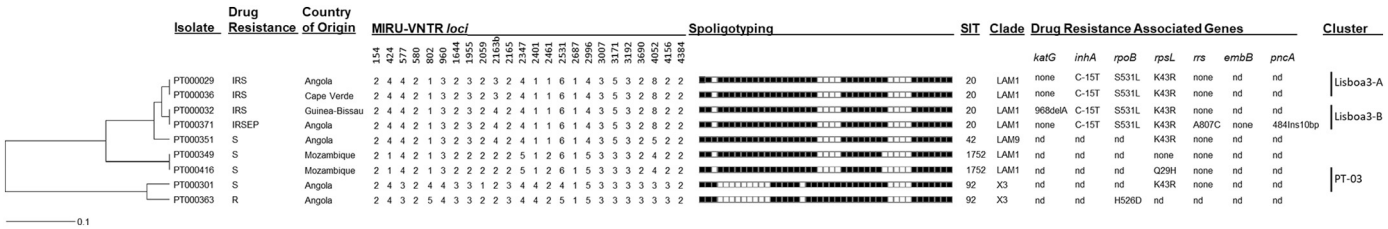

Fig. 2. Twenty-four loci MIRU-VNTR dendrogram comprising all nine drug resistant isolates included in the study. Two of three genetic clusters detected match the profile of Lisboa3-A and Lisboa3-B, two SIT20/LAM1 highly endemic genetic clusters to Portugal and associated with multidrug and extensively drug resistance, respectively. First-line drug resistance: I, isoniazid; R, rifampicin; S, streptomycin; E, ethambutol; and P, pyrazinamide.

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References

Andres, M., Gohring-Zwacka, E., Fiebig, L., Priwitzer, M., Richter, E., Rusch-Gerdes, S., Haas, W., Niemann, S., Brodhun, B., 2017. Integration of molecular typing results into tuberculosis surveillance in Germany-a pilot study. PLoS ONE 12, e0188356.

Brynildsrud, O.B., Pepperell, C.S., Suffys, P., Grandjean, L., Monteserin, J., Debech, N., Bohlin, J., Alfsnes, K., Pettersson, J.O., Kirkeleite, I., Fandinho, F., da Silva, M.A., Perdigao, J., Portugal, I., Viveiros, M., Clark, T., Caws, M., Dunstan, S., Thai, P.V.K., Lopez, B., Ritacco, V., Kitchen, A., Brown, T.S., van Soolingen, D., O'Neill, M.B., Holt, K.E., Feil, E.J., Mathema, B., Balloux, F., Eldholm, V., 2018. Global expansion of Mycobacterium tuberculosis lineage 4 shaped by colonial migration and local adaptation. Sci. Adv. 4 (eaat5869).

Couvin, D., Rastogi, N., 2015. Tuberculosis - a global emergency: tools and methods to monitor, understand, and control the epidemic with specific example of the Beijing lineage. Tuberculosis 95 (Suppl. 1), S177–S189.

Couvin, D., David, A., Zozio, T., Rastogi, N., 2018 Dec 26. Macro-geographical specificities of the prevailing tuberculosis epidemic as seen through SITVIT2, an updated version of the Mycobacterium tuberculosis genotyping database. Infect. Genet. Evol (Epub ahead of print; pii: S1567-1348(18)30969-9).

Demay, C., Liens, B., Burguiere, T., Hill, V., Couvin, D., Millet, J., Mokrousov, I., Sola, C., Zozio, T., Rastogi, N., 2012. SITVITWEB—a publicly available international multimarker database for studying Mycobacterium tuberculosis genetic diversity and molecular epidemiology. Infect. Genet. Evol. 12, 755–766.

Direcção Geral de Saúde, 2010. Relatório Para o Dia Mundial da Tuberculose: Ponto da Situação Epidemiológica e de Desempenho. In: Programa Nacional de Luta Contra a Tuberculose (PNT) Direcção Geral de Saúde. Ministério da Saúde, Lisbon.

Direcção Geral de Saúde, 2014. Portugal - Infeção VIH/SIDA e Tuberculose Em números - 2014. Direcção Geral de Saúde, Ministério da Saúde, Lisboa.

European Centre for Disease Prevention and Control/WHO Regional Office for Europe, 2017. Tuberculosis Surveillance and Monitoring in Europe 2017. European Centre for Disease Prevention and Control, Stockholm.

Gibson, A.L., Huard, R.C., Gey van Pittius, N.C., Lazzarini, L.C., Driscoll, J., Kurepina, N., Zozio, T., Sola, C., Spindola, S.M., Kritski, A.L., Fitzgerald, D., Kremer, K., Mardassi, H., Chitale, P., Brinkworth, J., Garcia de Viedma, D., Gicquel, B., Pape, J.W., van Soolingen, D., Kreiswirth, B.N., Warren, R.M., van Helden, P.D., Rastogi, N., Suffys, P.N., Lapa e Silva, J., Ho, J.L., 2008. Application of sensitive and specific molecular methods to uncover global dissemination of the major RDRio sublineage of the Latin American-Mediterranean Mycobacterium tuberculosis spoligotype family. J. Clin. Microbiol. 46, 1259–1267.

Groenheit, R., Ghebremichael, S., Svensson, J., Rabna, P., Colombatti, R., Riccardi, F., Couvin, D., Hill, V., Rastogi, N., Koivula, T., Kallenius, G., 2011. The Guinea-Bissau family of Mycobacterium tuberculosis complex revisited. PLoS ONE 6, e18601.

Instituto Nacional de Estatística, 2012. Censos 2011 Resultados Definitivos - Portugal. Instituto Nacional de Estatística, Lisboa.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J., 1997. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J. Clin. Microbiol. 35, 907–914.

Matteelli, A., Centis, R., Sulis, G., Tadolini, M., 2016. Crossborder travel and multidrugresistant tuberculosis (MDRTB) in Europe. Travel Med. Infect. Dis. 14, 588–590.

Perdigao, J., Silva, H., Machado, D., Macedo, R., Maltez, F., Silva, C., Jordao, L., Couto, I., Mallard, K., Coll, F., Hill-Cawthorne, G.A., McNerney, R., Pain, A., Clark, T.G., Viveiros, M., Portugal, I., 2014. Unraveling Mycobacterium tuberculosis genomic diversity and evolution in Lisbon, Portugal, a highly drug resistant setting. BMC Genomics 15, 991.

Perdigao, J., Silva, C., Diniz, J., Pereira, C., Machado, D., Ramos, J., Silva, H., Abilleira, F., Brum, C., Reis, A.J., Macedo, M., Scaini, J.L., Silva, A.B., Esteves, L., Macedo, R., Maltez, F., Clemente, S., Coelho, E., Viegas, S., Rabna, P., Rodrigues, A., Taveira, N., Jordao, L., Kritski, A., Lapa, E.S.J.R., Mokrousov, I., Couvin, D., Rastogi, N., Couto, I., Pain, A., McNerney, R., Clark, T.G., von Groll, A., Dalla-Costa, E.R., Rossetti, M.L., Silva, P.E.A., Viveiros, M., Portugal, I., 2018 Mar 17. Clonal expansion across the seas as seen through CPLP-TB database: a joint effort in cataloguing Mycobacterium tuberculosis genetic diversity in Portuguese-speaking countries. Infect. Genet. Evol (Published ahead of print; pii: S1567-1348(18)30102-3).

Rabna, P., Ramos, J., Ponce, G., Sanca, L., Mane, M., Armada, A., Machado, D., Vieira, F., Gomes, V.F., Martins, E., Colombatti, R., Riccardi, F., Perdigao, J., Sotero, J., Portugal, I., Couto, I., Atouguia, J., Rodrigues, A., Viveiros, M., 2015. Direct detection by the Xpert MTB/RIF assay and characterization of multi and poly drug-resistant tuberculosis in Guinea-Bissau, West Africa. PLoS ONE 10, e0127536.

Siddiqi, S., Rüsch-Gerdes, S., 2006. MGIT960 procedure manual for BACTECTM MGIT 960TM TB system (also applicable for manual MGIT). In: Mycobacteria Growth Indicator Tube (MGIT) Culture and Drug Susceptibility Demonstration Projects. Foundation for innovative new diagnostics Ed.

Supply, P., 2005. Multilocus variable number tandem repeat. In: Genotyping of *Mycobacterium tuberculosis*. Technical Guide. Institut de Biologie/Institut Pasteur de Lille.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S., Bifani, P., Kurepina, N., Kreiswirth, B., Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S., Skuce, R., Kremer, K., Locht, C., van Soolingen, D., 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of Mycobacterium tuberculosis. J. Clin. Microbiol. 44, 4498–4510.

Tavares, A.M., Fronteira, I., Couto, I., Machado, D., Viveiros, M., Abecasis, A.B., Dias, S., 2017. HIV and tuberculosis co-infection among migrants in Europe: a systematic review on the prevalence, incidence and mortality. PLoS ONE 12, e0185526.

van der Werf, M.J., Hollo, V., Kodmon, C., 2017. Multidrug-resistant tuberculosis and migration to Europe. Clin. Microbiol. Infect. 23, 578–579.

World Health Organization, 2013. Global Tuberculosis Report 2013. World Health Organization, Geneva.

Zammarchi, L., Casadei, G., Strohmeyer, M., Bartalesi, F., Liendo, C., Matteelli, A., Bonati, M., Gotuzzo, E., Bartoloni, A., Group, C.P.S., 2015. A scoping review of cost-effectiveness of screening and treatment for latent tubercolosis infection in migrants from high-incidence countries. BMC Health Serv. Res. 15, 412.