Differences in the robustness of clusters involving the *Mycobacterium tuberculosis* strains most frequently isolated from immigrant cases in Madrid

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Abstract

Tuberculosis cases infected by the same *Mycobacterium tuberculosis* (MTB) strain are considered to be clustered and involved in a transmission chain. Large clusters are assumed to represent active transmission chains in a population. In the present study, we focused on the analysis of large clusters defined by IS6110-restriction fragment length polymorphism (RFLP) typing in the immigrant population in Madrid. We identified 12 large clusters (involving 43% of the isolates) comprising 4–23 representatives. We proposed a gradient of epidemiological certainty for these large clusters. For a cluster to be considered robust and a good indicator of recent transmission, the MTB strain involved should not have been identified in a geographically and epidemiologically unrelated population and the cluster had to be re-confirmed by another highly discriminative molecular marker (MIRU-VNTR). The clusters that we discovered were classified into three categories: high, intermediate and low expected epidemiological value. In the largest cluster in the study (cluster M6; 23 representatives), failures by both criteria were identified: the representative seven-band RFLP pattern was also the most prevalent in the unrelated population (25 cases) and the cluster was fully split by MIRU-15, suggesting a lack of epidemiological value. The RFLP pattern representative of this cluster was also identified in 64 isolates from five countries in the Latin American genotype database, and again proved to be heterogeneous according to the MIRU-15 analysis. Specific analysis of large clusters, combined with the application of criteria for evaluating their robustness, could help identify uninformative clusters and target epidemiological resources towards those clusters with higher expected epidemiological value.

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Introduction

Molecular tools have been extensively applied to identify cases infected by the same *Mycobacterium tuberculosis* (MTB) strain [1–3]. These cases are defined as clusters and considered to be involved in the same transmission chain [4–8]. Although most of the clusters in a controlled population are small [8–10], analysis of those involving a higher number of cases is useful for the study of the bacterial and epidemiological features associated with active transmission [9,11,12].

Recent studies in Spain have revealed that, in addition to immigrant tuberculosis (TB) cases that appear to have been imported, a proportion of immigrant TB cases are caused by recent transmission after arrival in the host country [9,13–15]. In this situation, the specific analysis of immigrant clusters, especially large clusters, deserves attention. In studies that use clusters as indicators of recent transmission events, discrepancies with the epidemiological surveys have been found [4,16,17]. Even when advanced strategies are applied, epidemiological links are often not found for all the clusters in a population [8,17,18]. This suggests that not all clusters are informative from an epidemiological point of view.

Therefore, it would be extremely useful to define a gradient of epidemiological certainty for the clusters in a population.

We analysed the robustness and expected epidemiological informative value of large clusters among immigrants. For this purpose, we (i) compared the genotypes involved in clusters in a study in Madrid with the fingerprint data obtained from an unrelated population to try to identify fingerprint patterns that were prevalent in different settings and therefore not always indicators of recent transmission chains and (ii) checked the robustness of the restriction fragment length polymorphism (RFLP) clusters by applying a highly discriminatory genotyping tool: mycobacterial interspersed repetitive units–variable number of tandem repeats (MIRU-VNTR). Our proposal could open the way for attempts to target epidemiological resources towards the most probable active transmission events in complex populations, such as those with a high proportion of immigrant cases.

Materials and Methods

Study population

Madrid. The population of the Madrid area (comprising the city of Madrid and surrounding area) included 5 964 143 inhabitants in 2005, and there were 3197 TB cases diagnosed in the period 2004–2006. The incidence rates for the years 2004, 2005 and 2006 were 16.9, 19.1 and 18.5 cases per 100 000 inhabitants, respectively. Seventy-five percent of the TB cases (2397) were culture-positive.

Of the 2397 culture-positive cases diagnosed in the area of Madrid during the study period, 908 (37.9%) were immigrants and 1489 (62.1%) were autochthonous. The sample included all the culture-positive TB cases among immigrants from eight hospitals in the area during the study period (689 cases; 75.9% of the total number of culture-positive cases in immigrants) and all consecutive autochthonous cases from two hospitals (519 cases; 34.9% of the total number of culture-positive autochthonous cases). This sample was used to explore whether the genotypes prevalent in immigrants were also found in the autochthonous population.

Almería. The population covered by the study centres [health centres of the Regional Health Service (Servicio Andaluz de Salud) and the public network of mycobacteriology laboratories (Hospital de Poniente, CH Torrecárdenas and Hospital La Inmaculada)] ranged from 565 310 inhabitants in 2003 to 635 850 inhabitants in 2006 (average 598 388). The sample was composed of all patients with a microbiological diagnosis of TB during 2003–2006 [394 MTB isolates; 211 (53.6%) from immigrants and 183 (46.4%) from autochthonous cases].

Microbiological procedures

Clinical specimens were processed according to standard methods and inoculated on Lowenstein–Jensen slants and also in MGIT liquid medium (Becton Dickinson, Sparks, MD, USA) in some of the participating centres. Testing for susceptibility to isoniazid, rifampicin, streptomycin and ethambutol was performed according to standard methods.

Genotyping methods

In Madrid, 1208 MTB isolates from the hospitals involved in the study were received for genotyping and 92.1% of the isolates (1113) were genotyped.From Almería, 394 MTB isolates were received and 90.4% (356) were genotyped. Strains were analysed by IS6110-RFLP [19].

The large clusters (involving more than three cases) in Madrid were also typed by spoligotyping [20] and MIRU-VNTR by amplifying the 15 MIRU-VNTR loci as described previously [21], with some modifications. The final reaction volume of 50 μL used contained 1 μL (1 U) of Taq DNA polymerase (Roche Diagnostics, Basel, Switzerland) and 2 μL of dimethyl sulphoxide for Mix1 [580 (MIRU4), 2996 (MIRU26), 802 (MIRU40)] and Mix2 [960 (MIRU10), 1644 (MIRU16), 3192 (MIRU31)] and 6 μL for Mix3 [424 (Mtub04), 577 (ETRC), 2165 (ETRA)], Mix4 [2401 (Mtub30), 3690 (Mtub39), 4156 (QUB4156)] and Mix5 [2163b (QUB11b), 1955 (Mtub21) and 4052 (QUB26)]. One μl of the PCR products was mixed with 9 μL of formamide and 0.5 μL of GeneScan 2500 ROX size standard (Applied Biosystems, Foster City, CA, USA). DNA fragments were separated by capillary electrophoresis using ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Run parameters were created from the GeneScan36 POP4 default module, with a run voltage changed from 15 to 11 kV and run time set to 3600 s. Sizing of the PCR fragments was performed using GeneScan software (Applied Biosystems).

The MIRU-type was defined after combining the results for the 15 loci in the order: MIRU4, MIRU26, MIRU40, MIRU10, MIRU16, MIRU31, Mtub04, ETRC, ETRA, Mtub30, Mtub39, QUB4156, QUB11b, Mtub21 and QUB26.

Clustering analysis

Genotypic fingerprints were analysed using Bionumerics 4.6 (Applied Maths, St Martens-Latem, Belgium). Dendrograms were generated using the unweighted pair group method with arithmetic averages and the Dice coefficient for IS6110-RFLP analysis or the categorical coefficient for spoligotyping and MIRU-15 analysis.

RFLP clusters were defined for MTB isolates sharing 100% IS6110 fingerprint similarity. In two clusters, we tolerated clustering for those isolates that differed only in a low

molecular weight band (<1.10 kb) and showed high similarity (>93%) with the representative RFLP type. This decision was supported by the findings of previous studies [22,23]. Identical spoligotypes were required in addition to identical RFLP types for isolates with ≤5 IS6110 copies.

MIRU-15 clusters were defined when two or more isolates had identical MIRU types and variations in only one repetition at a single locus were tolerated. Families defined by spoligotyping were identified using the representative patterns from the SpolDB4 database [24].

Gradient of epidemiological certainty of large clusters

Large clusters in the study population of Madrid were categorized according to their expected epidemiologically informative value. For this purpose, we (i) compared the genotypes involved in clusters in a study in Madrid with the fingerprint data obtained from an unrelated population to try to identify fingerprint patterns that were prevalent in different settings and therefore not always indicators of recent transmission chains and (ii) checked the robustness of the RFLP clusters by applying a high discriminatory genotyping tool: MIRU-VNTR. Almería was the city selected as an unrelated population. Almería (Southeastern Spain) is 563 km from Madrid. There are only five other cities in Spain for which the distance from Madrid is greater than that of Almería. From an administrative point of view, Almería is part of an autonomous community different to Madrid.

The expected epidemiological value of the clusters was defined as:

1. High: when the isolates clustered by RFLP were genetically homogeneous by MIRU-15 and the representative RFLP type was not found in the unrelated population (Almería).
2. Intermediate: when at least half the isolates clustered by RFLP were genetically homogeneous by MIRU-15 or the representative RFLP type was found very infrequently in the unrelated population.
3. Low: when more than half of the isolates clustered by RFLP were split by MIRU-15 and/or the representative RFLP type was identified in more than one case in Almería

Latin American MTB genotype database

The database, located in Instituto Malbrán (Buenos Aires, Argentina), contains RFLP types from 3180 MTB clinical isolates obtained between 1992 and 2007 from cases diagnosed in Argentina (n = 2048) and another nine Latin American countries: Bolivia (n = 16), Brazil (n = 306), Chile (n = 35), Colombia (n = 236), Cuba (n = 10), Ecuador (n = 103), Paraguay (n = 195), Peru (n = 228) and Uruguay (n = 3).

Epidemiological survey

The epidemiological information was obtained from the Regional Registry of Tuberculosis in Comunidad de Madrid. For all patients, information was collected using a standardized protocol.

For the analysis of cluster M6 in Almería, we applied an advanced survey as described previously [13]. Briefly, transmission of TB was investigated using two information sources: data obtained using the standard approach (based on conventional contact tracing) and those obtained by applying two interviews. The objective of the first standardized interview was to collect complete data and photographs from the patients. The second interview aimed to compile new data and to search for potential epidemiological links based on nominal/photographic recognition between the clustered cases.

Geographical localization of clustered cases, when necessary, was performed by considering the household in which the case had lived for the previous 2 years. In cases with unstable households, the one that had been used for at least 3 months was included. The study was approved by the ethical committees of the involved institutions.

Results

Prevalent genotypes in immigrants

The first study aim was to identify the MTB strains that were most commonly isolated among immigrants. Of the 64 clusters identified in the immigrant population of Madrid during 2004–2006, (including 32.4% of the total number of cases), we found that most (52 clusters, 81.25%) involved one to three cases. Twelve clusters (4–23 representatives; 18.75% of the total number of clusters) were considered as large clusters because they involved four or more immigrant cases, and included a total of 88 cases (42.5% of the clustered cases) (Fig. 1). Two clusters included strains with ≤5 IS6110 copies (2 and 3 IS6110 copies), given that they were confirmed to share the same spoligotype. None of the 12 clusters revealed transmission of a drug-resistant strain, although some individual cases of drug resistance were identified (one isoniazid-ethambutol-streptomycin-resistant isolate in cluster M5, one isoniazid-rifampicin-pyrazinamide-resistant isolate in cluster M8, and one isoniazid-resistant isolate, one isoniazid-streptomycin-resistant isolate and one multiresistant isolate in cluster M6). Only two of the large clusters were uninational (with Ecuadorians and Romanians as the only nationalities involved), whereas the remaining ten clusters were multinational, involving cases from two to seven nationalities (Fig. 1). Spoligotyping revealed the

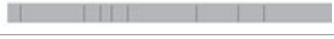
RFLP	RFLP type	Genetic lineage	N Immigrant cases	Country of origin	Cluster type	N Autochthonous cases	Mixed cluster	N Total
M30		**LAM5**	**4**	Peru, Morocco	**Multinational**	2	Yes	**6**
M68		**Unknown ST 106**	**4**	Ecuador	Uninational	0	No	**4**
M45		**H1var**	**7**	Romania	Uninational	0	No	**7**
M17		**LAM**	**5**	Ecuador, Morocco, Colombia, Peru, Dominican Rep.	**Multinational**	1	Yes	**6**
M14		**H3var**	**7**	Ecuador, Guinea, Morocco, Libia, Mali, Dominican R.	**Multinational**	10	Yes	**17**
M39		**LAM9**	**4**	Ecuador, Morocco	**Multinational**	0	No	**4**
M20		**H3**	**4**	Ecuador, Romania, Mexico	**Multinational**	0	No	**4**
M5		**H3var**	**10**	Guinea, Morocco, Ecuador, Argentina, Poland, Portugal	**Multinational**	4	Yes	**14**
M1		**H/H3**	**6**	Romania, Ecuador, Argentina	**Multinational**	1	Yes	**7**
M8		**X3**	**8**	Ecuador, Peru	**Multinational**	0	No	**8**
M85		**X3**	**6**	Ecuador, Colombia, Peru	**Multinational**	1	Yes	**7**
M6		**H3/ H3var**	**23**	Morocco, Peru, Ecuador, Paraguay, Bolivia, Poland, Romania	**Multinational**	5	Yes	**28**

FIG. 1. Representative features from the large clusters involving immigrants in Madrid. RFLP, restriction fragment length polymorphism.

absence of Beijing isolates and an involvement of the lineages: Haarlem (six clusters), LAM (three clusters) and X3 (two clusters).

In a second step, autochthonous cases were added to the analysis to determine whether the MTB strains involved in the large immigrant clusters, which were frequently shared by cases from different nationalities, were also found to infect autochthonous cases. Seven of the 12 large immigrant clusters also included autochthonous cases (from 1 to 10 cases), leading to mixed clusters (Fig. 1).

Informative value of large clusters

The next study objective was to analyse the robustness, and thus the expected value, of large immigrant clusters as potential indicators of true recent transmission events or, by contrast, whether some of them might be less informative. For a cluster to be considered a good indicator of recent transmission, we considered that it should fulfill two conditions: (i) the strains involved were not identified in a geographically and epidemiologically unrelated population, assuring that they were not prevalent strains in different settings, and (ii) clusters were re-confirmed when other, highly discriminative molecular markers were applied. The unrelated population selected was Almería, in southeastern Spain, and MIRU-VNTR (MIRU-15) was used for genotyping.

When the large clusters were double-checked, both conditions were fulfilled in five clusters (M30, M68, M45, M17 and M14); therefore, they were considered to be of high informative value from an epidemiological perspective (Fig. 2). Two of them were uninational clusters (M68 and M45) and the remainder were mixed clusters.

In four clusters (M39, M20, M1 and M8), we found an intermediate situation (Fig. 2): (i) cluster M39 was homogeneous by MIRU-15 and only one matched case was found in Almería and (ii) in the remaining clusters, no matches with other cases in the population of Almería were found and, although MIRU-15 discriminated some representatives, most of them were shown to be genotypically homogeneous. We considered that these four clusters might still have some informative value.

Finally, for the remaining three clusters (M5, M85 and M6), a higher number of matched cases were found in Almería or a marked genotypic heterogeneity was revealed by MIRU-15 (Fig. 2). In one of these clusters (M6; belonging to the Haarlem lineage), the largest in the study, including 23 cases from seven different countries [Bolivia (n = 3), Ecuador (n = 7), Paraguay (n = 1), Peru (n = 7), Poland (n = 1), Romania (n = 3) and Morocco (n = 1)] and five autochthonous cases, none of the conditions were fulfilled: (i) M6 RFLP-type was identified in 25 cases in Almería (Fig. 2) and (ii) the cluster was fully split by MIRU-15, both in Madrid (in five subclusters from 2 to 8 cases and seven unclustered cases) (Fig. 3a) and in Almería (in five subclusters from 2 to 10 cases and seven unclustered cases) (Fig. 3b). These findings suggest a low informative value for these RFLP-defined clusters.

Cluster	RFLP type	N Almeria	MIRU-15 type	N	Expectede pidemiological value
M30			2 6 4 4 3 3 2 4 2 2 5 2 3 2 5		High
M68			2 8 4 5 2 5 4 4 2 2 7 2 3 2 5		High
M45			2 5 3 4 3 3 1 3 3 3 3 3 5 3 7		High
M17			2 4 3 4 2 2 2 3 3 4 3 3 6 4 8 2 4 2 4 2 2 2 3 3 4 3 3 6 4 8	(5) (1)	High
M14			2 5 3 4 3 3 2 3 3 4 3 3 5 2 4		High
M39		1 Argentina	2 3 3 4 3 3 4 4 2 2 1 2 2 2 6		Intermediate
M20			3 5 3 6 3 3 2 3 3 4 4 2 5 3 7 3 5 3 6 3 3 2 3 3 4 2 2 5 3 7	(3) (1)	Intermediate
M1			2 5 3 5 3 3 2 3 3 4 3 1 7 3 4 2 5 3 5 3 3 2 3 3 4 3 3 4 2 7	(5) (2)	Intermediate
M8			2 4 2 4 3 3 2 3 3 4 3 3 7 4 6 2 4 2 4 3 3 2 3 3 4 3 3 7 3 6 2 4 2 4 3 3 2 3 3 4 3 3 2 4 6 2 4 2 4 3 3 2 3 3 4 3 3 5 4 6 2 4 2 4 3 3 2 3 3 4 3 3 7 4 5	(4) (1) (1) (1) (1)	Intermediate
M5		3 Spain (1) Senegal (1) Sweden (1)	2 5 3 4 3 2 2 3 2 4 3 3 4 3 5		Low
M85			2 4 2 4 3 3 4 3 3 4 3 3 4 3 5 3 6 2 4 2 4 3 3 4 3 3 4 3 3 4 3 6 3 6 2 4 2 4 3 3 24 3 4 4 3 3 4 6 2 4 2 4 3 3 2 3 3 4 3 3 6 3 5 2 4 2 4 3 3 2 3 3 4 3 3 7 4 6 1 4 2 4 3 3 2 3 3 4 3 3 7 4 6	(1) (1) (1) (1) (2) (1)	Low
M6		25 Romania (3) Senegal (1) Spain (16) Ecuador (2) Bolivia (1) Morocco (2)	See Figure 3		Low

FIG. 2. Distribution of the large clusters involving immigrants in categories of expected epidemiological value (high, intermediate and low). The number of matches identified in Almeria, and the mycobacterial interspersed repetitive units (MIRU) type for the representatives of each of the large clusters, are shown. RFLP, restriction fragment length polymorphism. MIRU-15 loci showing differences are highlighted in colour.

After distributing the large clusters among different categories according to our criteria for their informative value, we sent our proposal to the epidemiologists to check the epidemiological support identified in them. For the five clusters considered to be of high informative value (M30, M68, M45, M17 and M14), epidemiological links were found in all but M17. In clusters M68 and M45, familial links were identified; in M30, links were defined around a common risk factor (IVDU); and, finally, in M14, close relationships were found.

For the clusters in the intermediate category, which fulfilled our criteria to a large extent, although not completely, links were identified in two of the four clusters (M39 and M1). In M39, relationships among three Moroccan cases were defined as probable, whereas, in M1, proven links between two Romanian cases were found. It should be noted that epidemiological links were found between cases who had been considered grouped by MIRU-15 analysis.

Finally, for the three clusters not fulfilling our criteria (M5, M85 and M6), links were only partially found in six out of the 28 cases in cluster M6; between two spanish cases and among four cases of a peruvian family. Interestingly, the cases demonstrating epidemiological links had been considered grouped by MIRU-15 analysis (subclusters two and four; Fig. 3a).

With regard to cluster M6, the largest in study, which showed the lowest expected informative value according to

(a) MIRU15 LOCI

Cluster	580	2996	802	960	1644	3192	424	577	2165	2401	3690	4156	2163b	1955	4052	SpolDB4 Family	Country of origin
1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	7	H3	Bolivia
1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	7	H3var	Ecuador
1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	7	H3	Ecuador
1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	7	H3	Ecuador
1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	7	H3var	Bolivia
1	2	5	4	5	3	3	2	3	3	4	3	3	5	2	7	H3	Peru
1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	6	H3var	Paraguay
1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	6	H3var	Ecuador
2	2	5	3	5	3	3	2	3	3	4	3	3	3	2	7	H3	Poland
2	2	5	3	5	3	3	2	3	3	4	3	3	3	2	7	H3	Ecuador
2	2	5	3	5	3	3	2	3	3	4	3	3	3	2	7	H3	Peru
2	2	5	3	5	3	3	2	3	3	4	3	3	3	2	7	H3	Peru
2	2	5	3	5	3	3	2	3	3	4	3	3	3	2	7	H3	Bolivia
2	2	5	3	5	3	3	2	3	3	4	3	3	3	2	6	H3	Peru
	2	5	3	5	3	3	2	3	3	4	4	3	5	2	6	H3	Peru
3	2	5	3	5	3	3	2	3	3	4	3	3	7	3	6	H3	Romania
3	2	5	3	5	3	3	2	3	3	4	3	3	7	3	6	H3	Peru
	2	3	3	5	3	3	2	3	3	4	3	3	4	2	4	H	Peru
	2	5	3	5	3	3	3	3	3	4	3	3	8	2	6	H3	Spain
	2	5	3	5	3	3	3	3	3	4	3	3	6	2	4	H3	Morocco
	3	5	3	5	3	3	3	2	3	4	3	3	6	2	6	H3	Spain
	2	5	3	5	3	3	3	2	3	4	3	3	7	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
	2	5	3	5	3	2/3	2	3	2	4	3	4	4	2	7	H3	Ecuador
5	2	5	1	4	2	4	2	3	2	4	3	3	3	3	6	H3	Romania
5	2	5	1	4	2	4	2	3	2	4	3	3	3	3	6	H3	Romania
5	2	5	1	4	2	4	2	3	2	4	3	3	3	3	6	H3	Ecuador

(b) MIRU15 LOCI

Cluster	580	2996	802	960	1644	3192	424	577	2165	2401	3690	4156	2163b	1955	4052	SpolDB4 family	Country of origin
	2	5	3	5	3	3	2	3	3	4	3	3	2	3	5	H3	Romania
	2	5	3	5	3	3	2	3	3	4	3	3	7	3	5	H1	Senegal
	2	5	3	5	3	3	2	3	3	4	3	3	6	2	5	H3	Spain
1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	7	H3	Ecuador
1	2	5	3	5	3	3	2	3	3	4	3	3	5	2	7	H3	Bolivia
	2	5	3	5	3	3	2	3	3	4	3	3	2	2	7	H3	Ecuador
2	2	5	3	5	3	3	2	3	3	4	3	3	7	4	6	H3	Romania
2	2	5	3	5	3	3	2	3	3	4	3	3	7	4	6	H3	Romania
3	2	5	3	5	3	3	3	3	3	4	3	3	6	2	4	H3	Morocco
3	2	5	3	5	3	3	3	3	3	4	3	3	6	2	4	H3	Morocco
	2	5	4	5	3	3	3	3	3	4	3	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
4	2	5	3	6	3	3	2	3	3	4	2	3	6	2	6	H3	Spain
	2	5	4/3	6	3	3	2	3	3	4	4/3	3	6	2	6	H3	Spain
5	2	5	3	4	3	3	2	3	3	4	2	3	4	3	3	H3	Spain
5	2	5	3	4	3	3	2	3	3	4	2	3	4	3	3	H3	Spain
	2	5	3	5	3	4	2	4	3	4	5	3	4	3	7	H1var	Spain

(c) MIRU15 LOCI

Cluster	580	2996	802	960	1644	3192	424	577	2165	2401	3690	4156	2163b	1955	4052	SpolDB4 family	Country of origin
1	2	5	3	5	3	3	2	3	3	4	3	3	4	2	7	H3	Bolivia
1	2	5	3	5	3	3	2	3	3	4	3	3	4	2	7	H3	Ecuador
	2	5	3	5	3	3	2	3	3	4	3	3	7	2	7	H3	Latinamerican
2	2	5	3	5	2	3	2	3	3	4	3	3	5	2	7	H3	Bolivia
2	2	5	4	5	3	3	2	3	3	4	3	3	5	2	7	H3	Peru
2	2	5	3	5	3	4	2	3	3	4	3	3	5	2	7	H3	Ecuador
2	2	5	3	5	3	3	2	3	3	4	3	3	5	2	7	H3	Ecuador
2	2	5	3	5	3	3	2	3	3	4	3	3	5	2	7	H3	Bolivia
2	2	5	3	5	3	3	2	3	3	4	3	3	5	2	7	H3	Bolivia
3	2	5	3	5	3	3	2	3	3	4	3	3	5	2	6	H3	Peru
3	2	5	3	5	3	3	2	3	3	4	3	3	5	2	6	H3	Peru
3	2	5	3	5	3	3	2	3	3	4	3	3	5	2	6	H3	Latinamerican
	2	5	3	5	3	3	2	3	3	4	3	1	5	2	6	H3	Peru
4	2	5	3	5	3	3	2	3	3	4	3	3	6	2	6	H3	Bolivia
4	2	5	3	6	3	3	2	3	3	4	3	3	6	2	6	H3	Latinamerican
4	2	5	3	6	3	3	2	3	3	4	3	3	5	2	6	H3	Argentina
	2	5	3	5	3	4	2	3	3	4	3	3	4	2	5	H3	Latinamerican
	2	5	3	5	3	4	3	3	3	4	4	3	5	2	7	H3	Ecuador
	2	5	2	5	3	3	1	3	3	4	4	3	5	3	5	H3	Argentina

FIG. 3. Analysis of isolates sharing the M6 restriction fragment length polymorphism type in the Madrid (a), Almería (b), and Latin American (c) samples. The figure shows the mycobacterial interspersed repetitive units (MIRU) type, SpolDB4 family and country of origin of the corresponding cases. MIRU type clusters are indicated in grey. Alleles showing single locus variations with the other representatives in the MIRU cluster are shown in light grey. Orphan MIRU types are shown in white.

our criteria, we decided to analyse it in more detail, aiming to explore the meaning of the marked subdivision observed when MIRU-15 was applied. The analysis was also performed in Almería taking advantage of the availability of detailed epidemiological information on the cases, as a result of a standardized interview protocol used with the clustered cases, and supported by nominal/photographic recognition of the cases [13]. The first interesting observation was that seven cases were orphans by MIRU; second, the new subclusters fitted the nationality of the cases (Fig. 3b), with five subclusters grouping together Spanish, Moroccan, Romanian and Bolivian/Ecuadorian cases. Lastly, the geographic distribution of the cases was more consistent with the clustering patterns offered by the analysis by MIRU-15 (Fig. 4). Close locations between the cases included in MIRU-15-defined subclusters were found (subclusters 1, 2 and 3; Fig. 4). In subcluster 4,

FIG. 4. Geographic distribution of the cases infected by M6 isolates in Almería. The subclusters identified by mycobacterial interspersed repetitive units (MIRU) within the restriction fragment length polymorphism-defined M6 cluster are shown as numbers. White squares represent the cases clustered by MIRU. Black squares represent cases shown as orphan (o) by MIRU.

although three cases were geographically unrelated, most of the cases (7/10) were also geographically close (Fig. 4).

Initially, the epidemiological meaning of the RFLP-defined M6 cluster in Almería was not found and links were only identified between four of the 25 cases (two Romanian cases and two Spanish cases). Later, when the M6 cases were reanalysed according to the new distribution of cases in five subclusters offered by MIRU-15, we observed: (i) no links for the cases considered as orphan by MIRU-15 and (ii) links in eight cases included in three of the five MIRU-defined subclusters (subclusters 2, 4 and 5). Again, links were identified only between cases sharing a MIRU-defined subcluster, but never between cases from different subclusters.

Finally, we evaluated whether the RFLP pattern defining M6 was also prevalent in other countries. Sixty-four isolates from the Latin American MTB genotypes database were found to match with it (2% of the isolates). The cases infected with a strain sharing the M6 genotype corresponded to entries from Argentina (23 cases from both autochthonous and immigrant cases from Peru and Bolivia), Peru (18 cases), Colombia (13 cases), Paraguay (nine cases) and Ecuador (four cases). Spoligotypes were available for 32 of the 67 matched cases, and they all corresponded to the Haarlem lineage. A selection of 19 isolates representative of those with M6 genotype in Latin America were available for MIRU typing, and MIRU-15 again fully split the representatives sharing the M6 pattern into four subclusters and five orphan strains (Fig. 3c).

Discussion

The application of molecular tools to analyse MTB isolates allows the identification of clusters of cases infected by the same strain. These clusters are usually considered to belong to the same transmission chain, except for RFLP types, which are prevalent on a country-wide scale, and cannot imply epidemiologically linked cases [25,26]. Clusters in a population with efficient TB control programmes are generally small (two or three cases). A recent report from the Netherlands [10] found that 83% of clusters over an 11-year period had two or three cases. However, a percentage of cases belong to larger clusters, which are interesting to analyse in detail because they provide us with information about potential highly transmissible strains, or about the risk factors linked to active transmission chains [5,10,11].

In Spain, it has been found that, in addition to importation, recent transmission events play a role in the immigrant population [9,13,27]. Nineteen percent of the immigrant clusters (involving 42.5% of the MTB isolates) were found to be large and most of these were multinational and/or mixed, consistent with the high transmission permeability identified in Madrid [15].

Surveying the strains involved in active transmission chains may help identify 'hot-spot' transmission contexts [8,28–30]. Epidemiological surveys, which are often limited, could benefit from the identification of contexts that must be specially

targeted when they are expected to correspond to active recent transmission events. However, clusters are not always indicators of recent transmission, and epidemiological links often cannot be established in some cases, even after applying refined analytical methods [13,18]. RFLP-defined clusters without identifiable epidemiological links could also be considered to be the result of: (i) casual contacts that are not easily documented; (ii) independent coincidental infections by a prevalent circulating strain; and/or (iii) insufficient discriminatory power of the genotyping technique to reveal subtle genotypic differences among the clustered isolates. Different studies have attempted to refine the molecular and epidemiological analysis aiming to intensify the correlation between the identification of clusters and the existence of epidemiological links between the clustered cases [31–34].

Epidemiological information is often insufficient to enable us to confirm clusters as indicators of recent transmission. In this situation, it would be useful to define a gradient of expected informative relevance based on genotyping findings for clusters involving more than three cases in order to label them according to the degree of epidemiological certainty. Several studies [32,33,35] have shown that certain RFLP-defined clusters are split by MIRU-VNTR and that the new distribution of cases by MIRU-VNTR has an epidemiological significance. Considering this, and with the aim of establishing an epidemiological value gradient, we analysed two conditions that should be fulfilled for a cluster to be considered a good indicator of a recent transmission event: (i) the cluster can be reconfirmed when a second highly discriminatory molecular marker was applied, ruling out a lack of discrimination in defining clusters, and (ii) the strain involved in the cluster cannot be found in a geographically and epidemiologically unrelated population, which would reinforce its role in true recent transmission events, instead of coincidental independent infections by a prevalent widespread strain.

After considering the MIRU-VNTR data and the analysis of genotypes in the unrelated population, we classified the large clusters in Madrid into three categories, according to their expected informative value, and with no knowledge of the epidemiological data. To evaluate the certainty of this distribution, we later checked whether epidemiological links were found between the clustered cases.

For the five clusters that fulfilled our criteria for being considered informative, links were found in four of them, which supported our decision to consider them as being epidemiologically informative. Regarding the intermediate and low informative value categories, we found only some links and always between cases matched by the second-line MIRU-VNTR analysis.

From these observations, and for a RFLP-defined cluster that is also genotypically homogeneous by MIRU-15, it appears that the finding of matches with cases from another population does not weaken its epidemiological value to the same degree as in a cluster that is markedly subdivided by MIRU-15. Madrid (Central Spain) and Almería (Southeastern Spain) are distant enough (563 km) to insure that a general interaction between the populations will not occur. Almería is a city of entrance of immigrants into Spain from their countries of origin, and they later move to other cities (personal communication, Immigration and Labour Market Information, 2009; Permanent Observatory for immigration, Work and Immigration Ministry). Immigrants in Almería work mainly in the agriculture sector, which means that their later movements involve agricultural areas (i.e. that do not include Madrid). Among a sample of 500 immigrants in Almería, only 8.8% had stayed in Madrid, and just half of them for a period longer than 3 months. Also, in the context of the refined epidemiological survey carried out in Almería to analyse transmission patterns, we selected the biggest cluster in our study and 23 cases involved in the cluster were specifically asked whether they had stayed in Madrid. Only one had lived in Madrid but not during the period of illness.

However, mobility in immigrant cases is higher than in the autochtonous population and it is possible that some limited interactions happen. In this sense, the finding of epidemiological links in two clusters (M39 and M5), which were initially considered to have an intermediate or low epidemiological value because of the existence of matches with cases in Almería, led us to analyse the true meaning of these matches in more detail. We selected the cluster M5 for this analysis. Initially, the standard epidemiological survey found no links between three cases in Almería sharing the M5 genotype**.** In a second step, after applying an advanced system developed by our group to analyse recent transmission [13] which involves standardized interviews of the clustered cases and using nominal and photographic recognition between the cases to reveal epidemiological links, it was possible to detect that two of these three cases had spent several months in Madrid during the study period. This finding indicated that, at least for the M5 cluster, matches between Madrid and Almería could have an epidemiological significance. It also suggests that, for clusters involving immigrants, which are genotypically homogeneous both by RFLP and MIRU-15, the finding of matches in an independent population is possibly insufficient to decrease the certainty of the cluster.

One of the clusters in our analysis, the largest in the study (M6), was considered to have the lowest epidemiologically informative value. It was the only one with none of the

required features: frequent matches were found in Almería, and the cluster was fully split by MIRU. We studied in detail the meaning of this subdivision when MIRU was applied. This cluster had initially been investigated in Almería by applying the previously mentioned advanced system for an analysis of recent transmission [13]. It was quite complex because of its dimensions and the links supporting M6 were not found, probably owing to the poor identification of cases sharing the cluster when 50 names/photographs (25 related cases and 25 unrelated controls) were shown to the cases involved. Nevertheless, the new distribution of cases according to MIRU-15 data fits with the nationalities of the cases involved, and the geographical distribution of the cases was more consistent.

When the same advanced survey was applied to the subdivisions of the cluster defined by MIRU-15, although not all MIRU-defined subclusters were epidemiologically supported, we observed that links were found only between cases sharing a MIRU-defined subcluster but never between cases from different subclusters. The findings for cluster M6 could represent new examples of the higher epidemiological precision of MIRU-15 when defining clusters, as reported previously [32,35,36].

The seven-band pattern defining this M6 cluster belongs to the Haarlem family and is common in other settings [21,32,33,37,38]. Haarlem clusters have also been split by MIRU [36], although not always [33]. In the Latin American MTB genotype database, the M6 genotype was frequently detected in immigrants from different countries. In Paraguay, this Haarlem genotype was named 'Tacumbú' and its presence has increased over time [37]. This 'Tacumbú' genotype showed a clonal structure, with the isolates sharing an inedit Haarlem 3 spoligotype (SIT2643) and the same MIRU-12-type [37]. In the present study, one of the cases involved in the M6 cluster corresponded to a Paraguay-born patient who was the only one showing the SIT2643 spoligotype. Similarly, homogeneous clones according to MIRU data, and sharing the M6 RFLP pattern, are expected to be present in other countries and could have been imported into Spain. This might explain the aggregation of nationalities identified in the present study with respect to the different MIRU types identified within the M6 RFLP-defined cluster.

Our findings indicate the existence of a common RFLP pattern (M6) worldwide and suggest that this genotype has some kind of adaptative advantage. The presence of this ubiquitous RFLP pattern could lead to false identification of recent transmission among the cases involved if only RFLP data are used.

The present study demonstrated the usefulness of specifically analysing large clusters to obtain information about the transmission dynamics of complex populations, such as ours, with a high proportion of immigrant cases. However, to optimize the efficiency of the epidemiological survey guided by molecular information, the data from the present study emphasize the need to examine the analysis critically to identify those clusters with the highest informative value and to identify others that could add confusion to the analysis. The latest generation of genotyping tools, such as VNTR-based designs, have also proven useful for revealing imprecise clusters of cases defined by standard analysis based on RFLP. Furthermore, sharing MTB fingerprints between the groups involved in genotyping MTB from independent populations could prove useful for identifying clusters with a low informative value, as a result of coincidental infections by prevalent strains. The data obtained in the present study suggest that the finding of matches between independent populations by itself should not weaken the certainty of clusters to the same degree as that of the subdivision of a cluster by second-line MIRU-based typing. For a refined analysis of active transmission events, the establishment of a gradient of expected informative value for clusters appears useful, especially in circumstances where epidemiological data from patients are scarce, survey resources are limited, and/or the efficiency in managing the affected population is low, as in the case of our immigrant population. This refinement in the analysis of clusters could enable us to target those clusters that are particularly fruitful for identifying active transmission settings.

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Transparency Declaration

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