ELSEVIER

Molecular and Cellular Probes 19 (2005) 236–244

MOLECULAR AND CELLULAR PROBES

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Comparison between spoligotyping and IS*6110* restriction fragment length polymorphisms in molecular genotyping analysis of *Mycobacterium tuberculosis* strains☆

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Received 21 June 2004; accepted for publication 5 January 2005
Available online 17 March 2005

Abstract

Spoligotyping was compared with RFLP fingerprinting analysis in the identification of *Mycobacterium tuberculosis* strains. Spoligotyping sensitivity was 97.6% with a specificity of 47%. The global probability for two strains clustered with spoligotyping to be clustered also with RFLP analysis was 33%; the probability for two strains clustered with RFLP analysis to be clustered also with spoligotyping analysis was 95%. However, comparing the two methods in five outbreak episodes, full concordance was evidenced between spoligotyping and RFLP. Moreover, we evaluated the presence of our 17 largest spoligotyping clusters in spoligotyping databases from Caribbean countries, London and Cuba. Only five out of 17 patterns were present in all the cohorts. The conditional probability comparing spoligotyping and RFLP methods related to these patterns resulted in very low concordance (range from 2 to 38%). In conclusion, we confirm that spoligotyping when used alone overestimates the number of recent transmission and does not represent a suitable method for wide clinical practice application. However, it allows to get a first good picture of strain identity in a new setting and in more localized or confined settings, the probability of reaching the same result compared to RFLP was 100% confirming the usefulness of spoligotyping in the management of epidemic events, especially in hospitals, prisons and close communities.
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Keywords: Tuberculosis; Multidrug resistant; Molecular epidemiology; Bacterial typing

1. Introduction

The re-emergence of tuberculosis, observed both in developing and developed countries, gave rise to the need for rapid methods of differentiation among *Mycobacterium tuberculosis* strains and timely identification of the emergence of epidemic events [1–7].

Molecular epidemiological studies of the spread of tuberculosis has been extensively performed with the development of molecular methods [8–10]. Molecular typing is a valuable tool in epidemiological investigations of tuberculosis and identification of potential sources of

☆ This work was supported by a grant of the National Institute of Health (ISS), Minister of Health, 1% 2000, project 0AL/F and RF/101, Rome, Italy, and by a grant of the AHSI Company, Milan, Italy.

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doi:10.1016/j.mcp.2005.01.001

infection, both in the general population and in nosocomial settings [11–18].

The most commonly used genotyping analysis for tuberculosis is the restriction fragment length polymorphism (RFLP) analysis using IS*6110* as the molecular probe [10,12,14,19]. This technique is highly discriminatory and results are stable and reproducible, but it presents some difficulties. First, it requires large quantities of DNA obtainable only from prolonged cultures with a considerable delay (20–40 days) in results [20]. Second, it discriminates poorly among isolates with a low copy number of IS*6110* [21–23]. Thus secondary typing techniques are needed to differentiate such isolates [24]. Third, it is quite expensive and requires sophisticated software for analysis of typing patterns on gels and local and global genotyping databases may thus be not easily constructed [25,26].

Spoligotyping is a newer molecular typing method frequently used to discriminate between *M. tuberculosis* isolates. It is based on sequence variation within the direct repeat (DR) region of the *M. tuberculosis* genome [20]. One of its most important advantages is that this technique involves PCR amplification, so minimal quantities of DNA are required. This allows analysis based directly on clinical material, as for smear positive samples [20,23,27]. Thus spoligotyping results could be useful in cases of acute tuberculosis disease, rapid identification of outbreaks and laboratory cross contaminations [19,27–32]. Moreover, the spoligotyping method is cheaper than RFLP and requires less technical ability. Results are easily reproducible and stable, even if spoligotyping is less effective than RFLP in discriminating different genotypes [22,28–30,33]. Finally, spoligotype patterns can be read and recorded with a simple word processor program and local and global genotyping databases may thus be easily constructed [25,26].

The primary aim of this study was to evaluate the capability of spoligotyping to discriminate between different *M. tuberculosis* genotypes and to compare spoligotyping results with those obtained by using the IS*6110* RFLP analysis. We calculated sensitivity, specificity, positive and negative predictive value, and conditional probability of spoligotyping results. The analysis was performed in a heterogeneous patient population. The study included both sporadic cases and cases belonging to well characterized TB outbreaks. This enabled us to assess the potential effectiveness of spoligotyping in epidemiological practice.

The study also was intended to reveal whether data obtained in a determinate context could be extrapolated on a widespread geographic scale. To accomplish this, we performed a comparison between the spoligotyping versus RFLP results obtained in Italy and those on strains showing the same spoligotyping genotype found in other geographical contexts [27,29,32].

2. Material and methods

2.1. Mycobacterial strains collection

We conducted a prospective study on all the consecutive *M. tuberculosis* culture positive patients diagnosed in all the area of Milan and its Province in the period from 1993 to 2001. All the available *M. tuberculosis* strains were collected by the Microbiological Laboratory of the 'L. Sacco' Hospital, University of Milan and by the Laboratory of the 'Villa Marelli' Institute (VMI), Reference Center for Tuberculosis Control in Lombardy. Primary mycobacterial isolation was done using Lowenstein–Jensen slant cultures and radiometric method (BACTEC). Culture procedures, species identification and susceptibility tests, to isoniazid, rifampin, ethambutol, streptomycin and pyrazinamide were carried out according to standard methods [34].

2.2. DNA extraction from mycobacterial cultures

Mycobacteria were harvested from Stonebrink slopes, transferred into a microcentrifuge tube containing 400 μL of 1×Tris–EDTA (TE buffer) and incubated at 80 °C for 20 min. Fifty microliters of 10 mg/mL lysozyme were added and the mixture was incubated at 37 °C for at least 1 h. Seventy-five microliters of a mix containing 150 μL of 10 mg/mL proteinase K (Sigma Aldrich, WI, USA) and 2.1 mL of 10% sodium dodecyl sulfate (SDS) were added to the lysozyme-treated samples. After vortexing and incubating for 10 min at 65 °C, 100 μL of 5 M NaCl was added and subsequently 100 μl of *N*-cetyl-*N*,*N*,*N*,-trimethyl ammonium bromide (CTAB)/NaCl solution was added, with the aim of binding cell wall debris, denatured proteins and polysaccharides. After vortexing until the liquid content became white ('milky'), the mixture was incubated for 10 min at 65 °C. DNA was then extracted by the standard phenol–chloroform extraction method [34].

2.3. Genotypical analysis

Isolates of *M. tuberculosis* were genotyped by RFLP DNA fingerprinting using the IS*6110* probe as a genetical marker, as previously described by van Embden et al. [34].

Spoligotyping, a PCR method based on the polymorphic DR region containing 36-bp direct repeats and interspersed 35–41 bp variable spacer sequences, was performed on genomic DNA by standard method as previously described by Kamerbeek et al. [20].

2.4. PCR control procedures

Each PCR was performed with positive and negative controls by means of sterile procedures, following contamination-free guidelines to prevent false-positive results [35]. The chance of PCR contamination was minimized by physical separation of the amplified products from starting

materials. All pre-PCR handling of materials took place in a room separate from the PCR site (which had a circulation-free, sterile bench and UV lighting). Another room was dedicated to the processing and analysis of all of the amplification products.

2.5. Computer-assisted analysis of the patterns

Comparison analysis of the hybridization patterns obtained by spoligotyping and IS*6110* RFLP DNA fingerprinting was performed with a computer software program. We used the Dice Index for pattern comparison and the unweighted pair group method with arithmetic averages (UPGMA) (GelCompar Software-Version 4.1; Applied Maths, Kortrijk, Belgium).

2.6. Statistical analysis

The sensitivity of spoligotyping was calculated as the number of patients clustered by both spoligotyping and RFLP-IS*6110* probe methods, divided by the total number of patients clustered by RFLP-IS*6110* probe analysis. The specificity of spoligotyping was calculated as the number of patients clustered neither by spoligotyping nor by RFLP-IS*6110* probe method, divided by the total number of patients not clustered by RFLP-IS*6110* probe method. We remind that the sensitivity means the probability of being a true positive in a diagnostic test and the specificity means the probability of being a true negative in a diagnostic test.

The degree of concordance between the two clusterization methods (RFLP and spoligotyping) was obtained in two ways.

The first approach was to classify the data in a two-by-two contingency table, where each cluster is classified according to two binary variables: belonging/not belonging to a cluster according to RFLP and belonging/not belonging to a cluster according to spoligotyping. With this approach, it does not matter whether the cluster is the same or different. We calculated the probability of the data in each of the four cells. A Pearson chi-square test was performed to test the statistical significance of the association between the two binary variables. In the second approach, we tested the concordance between the two methods if two genotypes belonged to exactly the same cluster according to one clusterization method given that they belong to the same cluster using the other classification method. We calculated the conditional probability that two genotypes belong to the same cluster according to RFLP, given that they belong to the same according to spoligotyping classification cluster, and the probability that two observations classified in the same RFLP cluster belong to the same spoligotyping cluster.

We conducted the same analysis on the strains collected by other authors in London [29], Cuba [27] and Caribbean countries [32] limiting it to the conditional probability that two genotypes belong to the same RFLP cluster, given that they have the same spoligotyping pattern.

3. Results

A total of 700 consecutive isolates collected at 'L. Sacco' Hospital and 'Villa Marelli' Institute from 1993 to 2001 and identified as *M. tuberculosis* and *Mycobacterium bovis* by classical mycobacteriology procedures, were the subject of the present investigation. The isolates were first studied by spoligotyping and subsequently typed by the RFLP-IS*6110* probe analysis. Seven isolates were excluded from the final analysis because material was not available for RFLP analysis.

Among the studied isolates, nine *M. bovis* strains were found. All these strains exhibited the same one-band pattern at RFLP analysis (cluster no. 91) and were correctly identified by spoligotyping showing the characteristic genomic profile of the *M. bovis* species (cluster no. 17).

Ten *M. tuberculosis* strains belonging to the Beijing family were identified as cluster using spoligotyping (pattern 14) and a total of seven different RFLP patterns were found: one cluster of two isolates (Italian patients), one cluster of three isolates (Chinese patients) and five single isolates (two Chinese, two Italian and one Moroccan patients).

3.1. Comparison of spoligotyping and IS6110 RFLP

Among the 700 spoligotypes analyzed, a total of 240 different patterns were observed. One-hundred-seventy-six strains (25.1%) were found only once and were classified as sporadic patterns. The other 524 isolates (74.9%) were grouped in 64 different clusters shared by two or more strains: 25 clusters of two isolates each, 11 clusters of three isolates each, five clusters of four isolates each, two cluster of five isolates, two clusters of six isolates each, two clusters of seven isolates each, two clusters of eight isolates each, three clusters of nine isolates each, two clusters of 11 isolates each, and 10 clusters with more than 11 isolates (range from 12 to 70 each) (Fig. 1).

RFLP-IS*6110* analysis of 693 available isolates resulted in 446 different DNA fingerprints, of which 354 (51.1%) were found once representing sporadic patterns. Three-hundred-thirty-nine isolates (48.9%) were grouped in 92 clusters. The cluster sizes varied from 2 to 17. The results of cluster analysis with the two genotyping techniques are summarized in Fig. 1.

One-hundred-eighty-six isolates classified as unique with RFLP-IS*6110* were grouped in clusters when analyzed by spoligotyping and eight isolates classified as unique with spoligotyping were included in clusters when analyzed by IS*6110*-RFLP.

Eighty-six of the total 92 RFLP clusters into which the 693 samples were divided were correctly identified by

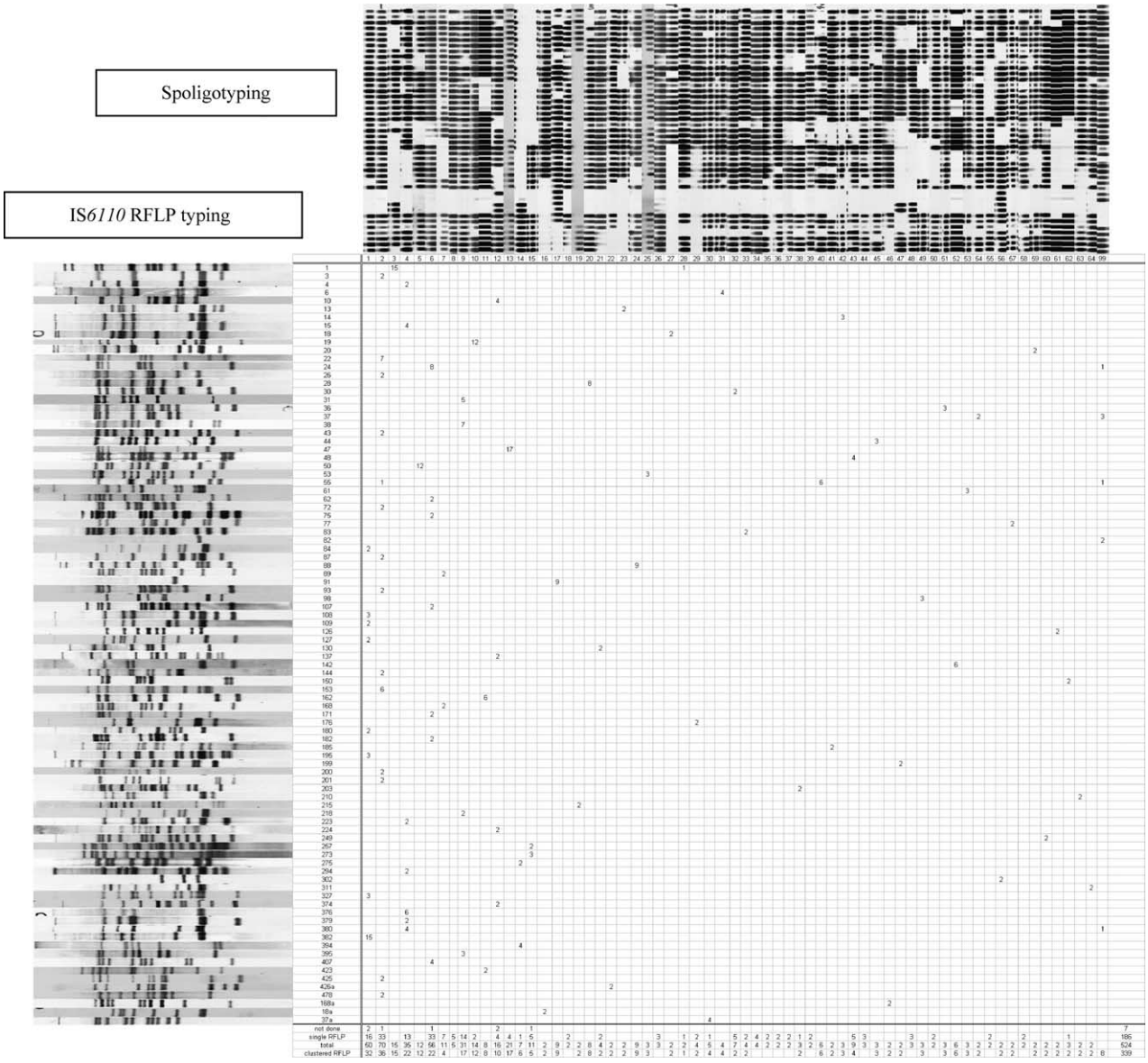


Fig. 1. Spoligotype patterns generated by clustered *M. tuberculosis* strains after computer analysis compared with the corresponding IS*6110* RFLP DNA fingerprints patterns.

spoligotyping. Five of 92 RFLP clusters (all these genotype patterns had more then five IS*6110* bands) were partially identified by spoligotyping (nos. 1, 24, 37, 55, 380) while one of 92 clusters, represented by a one-band IS*6110* RFLP pattern, was further correctly differentiated in two different sporadic patterns by spoligotyping (no. 82) (Fig. 1).

3.2. Statistical analysis

The associations of clustered spoligotypes with IS*6110* RFLP clustering, calculated by Pearson's chi-square test with Yates' continuity correction, resulted 183.52 (with one degree of freedom, p-value >0.001; 183.52 was obtained by performing chi square test on the contingency table shown in Fig. 1). Since many cells present few observations, Yates' correction has been applied, the latter basically consists on subtracting 0.5 from each observed value in a 2×2 contingency table). This result shows strong similarity between the results produced by the two different classification methods. Comparison between the two genotyping methods showed that spoligotyping sensitivity was 97.6% with a specificity of 47%. The global positive predictive value was 64% while the negative predictive value was 95.5%.

The positive predictive value indicates the probability of being a true positive given that the test is positive, on the other hand the negative predictive value is the probability of being true negative, given the diagnostic test is negative.

We therefore calculated for each observed spoligotyping cluster the conditional probability for two genotypes to

belong to the same RFLP cluster being identical with spoligotyping analysis.

The global probability for two strains clustered with spoligotyping to be clustered also with RFLP analysis was 33%; conversely the probability for two strains clustered with RFLP analysis to be clustered also with spoligotyping analysis was 95%. Only one of the six RFLP clusters not recognized by spoligotyping had a genotype with fewer than five bands (genotype with a single band, not *M. bovis*). Among the 25 spoligotyping clusters with two isolates each, 16 (64%) were identical both with spoligotyping and with RFLP analysis (conditional probability = 100%), while nine (36%) clusters were not confirmed by RLFP analysis (conditional probability = 0%).

Nine out of 22 spoligotyping clusters (41%) including from 3 to 7 isolates each showed a full correspondence between the two methods.

Only five of the 17 (29%) largest spoligotyping clusters (from 8 to 70 isolates each) identified with spoligotyping were not subdivided by RFLP. All these five clusters, defined by epidemiological investigation and molecular results, were related to known epidemiological TB outbreaks due to MDR-TB or *M. bovis* strains. The conditional probability calculated on the other 12 clusters ranged from 2 to 72.5%. The global conditional probability calculated on these 17 largest spoligotyping clusters was 25.8%. The results are shown in Fig. 2.

3.3. Comparison with other geographical contexts

In order to evaluate whether the relation between spoligotyping and IS*6110* RFLP was the same in other geographical areas, we analyzed the presence of the strains isolated in our 17 largest spoligotyping clusters (including from 8 to 70 isolates), comparing results with those from several spoligotyping databases built in other geographical contexts: London, Cuba and the Caribbean region.

Only five out of 17 patterns were present in the other three cohorts (London, Cuba and Caribbean) where they did not represent the most frequent patterns identified. In two cases the patterns were sporadic. One of the five patterns always present was the Beijing pattern. None of the five strains correlated in Italy with a known epidemic spread of tuberculosis (patterns that showed the highest correspondence between spoligotyping and RFLP, conditional probability = 100%), was present in the other different geographical contexts evaluated (Fig. 3).

On a total of 448 isolates from the Caribbean region typed between 1994 and 1999, 117 isolates (26%) were grouped in eight spoligotyping patterns also observed in Italy. The other nine patterns were not represented.

In London, 44 out of 167 isolates collected (26%), divided in seven spoligotyping clusters ranging from 2 to 20 isolates each, were detected also in our series.

Sixty-one out of 160 isolates (38%) collected in Cuba in 1994–1995, showed six different patterns identical to those present in our database. Two patterns were sporadic while four patterns were represented in clusters ranging from 7 to 20 isolates.

The IS*6110* RFLP analysis of the isolates grouped in clusters by spoligotyping and present outside of Italy is summarized in Fig. 3.

We calculated region by region the conditional probability comparing spoligotyping and RFLP methods. In the Caribbean region, the conditional probability ranged from 0 to 40% with a global conditional probability of 7.7%. In London, the conditional probability was 0 for all patterns except for nos. 2 and 15 (conditional probability, respectively, of 0.53 and 4.76%); the global conditional probability was 1%. In Cuba, the conditional probability ranged from 0 to 80% with a global conditional probability of 35.6%.

The conditional probability related to the five patterns present in all four cohorts was low (range from 2 to 38%). All these results are summarized in Fig. 3.

4. Discussion and conclusions

Considering the rising amount of tuberculosis cases in developing countries such as in industrialized ones, the emergence of even more drug resistant strains and in particular MDR-TB strains, and the occurrence of outbreaks in hospitals, prisons and close communities, molecular epidemiology investigation has become a primary objective [5,11–13,15,17–19].

Consequently, the standardization of new, cheap, easy and rapid typing methods, has become increasingly urgent [2,4,7,20,36].

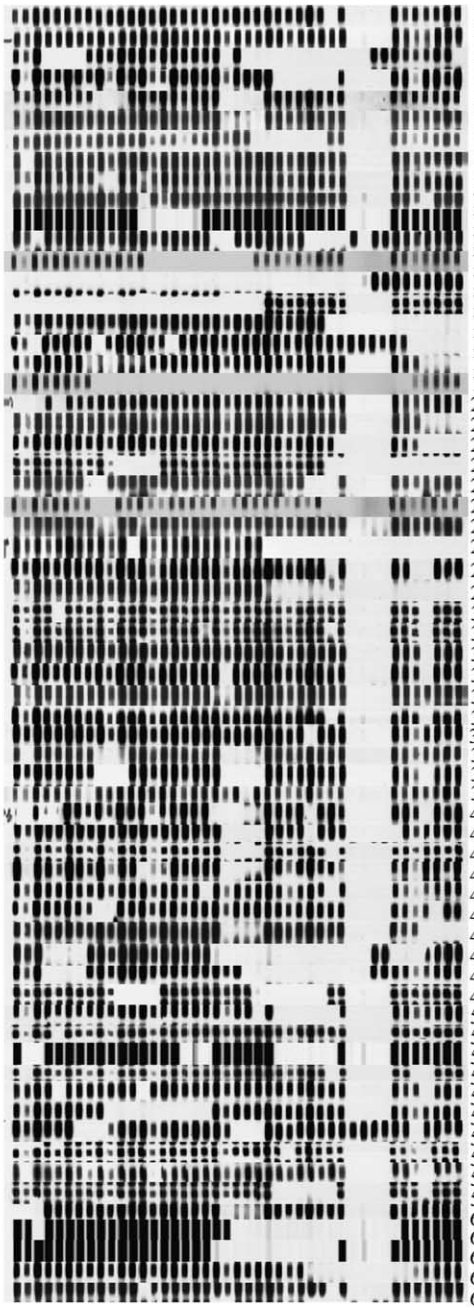
The primary aim of our study was to evaluate the capability of the spoligotyping method to discriminate between different *M. tuberculosis* genotypes, comparing results with those obtained by using the IS*6110* RFLP fingerprinting method, and to detect and quantify, case by case, the clustering error. We determined for this purpose sensitivity, specificity, positive and negative predictive value, and conditional probability of spoligotyping results.

This analysis allows an assessment of spoligotyping's potential usefulness in clinical and epidemiological practice.

Consequent to spoligotyping analysis, ¼ of the cases were sporadic, while ¾ were clustered. On the cases, IS*6110* RFLP analysis showed that only half of the cases were clustered and sporadic cases represented the other half. Spoligotyping overestimated the number of clustered isolates in our region by approximately 25% compared to IS*6110* RFLP analysis.

These data confirmed, as previously described, the low specificity of spoligotyping, although patients with clustered spoligotypes were significantly associated with IS*6110* clustering [22,23,33,36].

In general, the positive predictive value of spoligotyping with respect to IS*6110*-RFLP was low. This suggests that

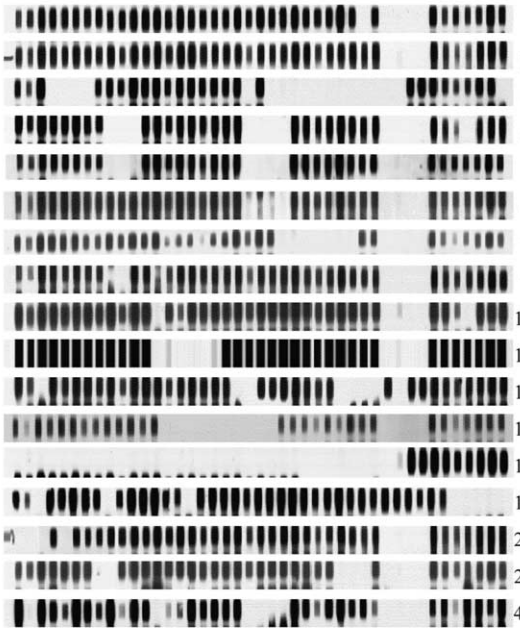


Spoligotype n.	Number of strains	N. of Single RFLP	N. of Clustered RFLP	RFLP-Spoligo concordance
1	48	16	32	0.10
2	69	33	36	0.02
3	15	0	15	1.00
4	35	13	22	0.05
5	12	0	12	1.00
6	55	33	22	0.02
7	11	7	4	0.03
8	5	5	0	0.00
9	31	14	17	0.07
10	14	2	12	0.72
11	8	0	8	0.57
12	14	4	10	0.09
13	21	4	17	0.64
14	10	5	5	0.08
15	7	1	6	0.33
16	2	0	2	1.00
17	9	0	9	1.00
18	2	2	0	0.00
19	2	0	2	1.00
20	8	0	8	1.00
21	4	2	2	0.16
22	2	0	2	1.00
23	2	0	2	1.00
24	9	0	9	1.00
25	3	0	3	1.00
26	3	3	0	0.00
27	2	0	2	1.00
28	2	1	1	0.00
29	4	2	2	0.16
30	5	1	4	0.60
31	4	0	4	1.00
32	7	5	2	0.04
33	4	2	2	0.16
34	4	4	0	0.00
35	2	2	0	0.00
36	2	2	0	0.00
37	2	2	0	0.00
38	3	1	2	0.33
39	2	2	0	0.00
40	6	0	6	1.00
41	2	0	2	1.00
42	3	0	3	1.00
43	9	5	4	0.16
44	3	3	0	0.00
45	3	0	3	1.00
46	2	0	2	1.00
47	2	0	2	1.00
48	3	3	0	0.00
49	3	0	3	1.00
50	2	2	0	0.00
51	3	0	3	1.00
52	6	0	6	1.00
53	3	0	3	1.00
54	2	0	2	1.00
55	2	2	0	0.00
56	2	0	2	1.00
57	2	0	2	1.00
58	2	2	0	0.00
59	2	0	2	1.00
60	2	0	2	1.00
61	2	0	2	1.00
62	3	1	2	0.33
63	2	0	2	1.00
64	2	0	2	1.00

Fig. 2. Comparison of spoligotyping and IS*6110* RFLP fingerprinting: quantification of the concordance between the two clusterization methods by calculating the conditioned probability for two genotypes to belong to the same RFLP cluster being identical with spoligotyping analysis.

spoligotyping alone is not a suitable method for wide clinical practice application or purely epidemiological studies. However, to increase the spoligotyping usefulness, an ideal time-saving screening system could be made up of the association of two PCR-based system. Different studies have already described the advantage of the association of spoligotyping with Spol-DRE [32], Spol-LM-PCR [37], Spol-VNTR [32,38], Spol-LM-PCR-VNTR [39] or MIRU-VNTR [40].

Moreover, the attempt to overcome this obstacle caused us to examine quantification of the error, finding a probability value for each spoligotype clustered to be confirmed with IS*6110*-RFLP method, thus showing the possible actual epidemiological usefulness of spoligotyping.

Spoligotype n.	RFLP - spoligotyping concordance			
	Italy (n.)	Cuba (n.)	Caribbean Region (n.)	London (n.)
 1	0.10 (48)	0.25 (16)	0.15 (32)	0.25 (4)
2	0.02 (69)	0.09 (16)	0.06 (40)	0.05 (20)
3	1.00 (15)	NA*	NA	NA
4	0.05 (35)	0.39 (7)	NA	0.20 (5)
5	1.00 (12)	NA	NA	NA
6	0.03 (55)	NA	0.12 (10)	0.33 (3)
7	0.04 (11)	1.00 (1)	0.50 (2)	0.33 (3)
9	0.07 (31)	1.00 (1)	0.33 (3)	0.33 (23)
10	0.72 (14)	NA	NA	NA
11	0.57 (8)	NA	NA	NA
12	0.10 (14)	NA	NA	NA
13	0.65 (21)	NA	NA	NA
14	0.09 (10)	0.81 (20)	0.33 (3)	0.50 (7)
17	1.00 (9)	NA	NA	NA
20	1.00 (8)	NA	NA	NA
24	1.00 (9)	NA	NA	NA
43	0.17 (9)	NA	0.52 (5)	NA

* NA: spoligotyping pattern not applicable in the cohort

Fig. 3. Representation of IS*6110* RFLP fingerprinting and spoligotyping concordance: comparison between the results obtained in Italy and those on strains showing the same spoligotyping genotype found in other geographical contests.

While literature has so far merely investigated specific methodological issues comparing different performances of several typing techniques [21–23,26–29,32], we aimed to evaluate the real impact of spoligotyping used in clinical setting for epidemiological studies of tuberculosis transmission.

The probability that two or more strains, clustered with spoligotyping analysis, also would cluster using IS*6110*-RFLP analysis was 100% in case of tuberculosis outbreaks.

In fact, all cases belonging to known tuberculous outbreaks have been correctly identified and clustered both with spoligotyping and with IS*6110*-RFLP analysis. These data confirmed the undoubted usefulness of spoligotyping, in case of epidemic spread of tuberculous infection, especially in hospitals, prisons and close communities in general, to rapidly and inexpensively identify new cases and to attribute them or not to the ongoing outbreak. Timeliness of information can allow prompt intervention to stop diffusion through the application of strict isolation measures. Moreover, in case of known MDR-TB outbreaks, an adequate anti-tuberculous therapy could be prescribed, on the basis of the typing results, long before traditional susceptibility test results would become available.

On the other hand, we found a very low probability of spoligotyping and IS*6110*-RFLP correspondence when the analyzed strains did not proceed from the same temporal or geographical context. Strain collected in different years and in different cities even if clustered with spoligotyping analysis often were determined to be sporadic after IS*6110*-RFLP analysis.

Moreover, this study reveals that data obtained in a determinate context could be hardly extrapolated on a widespread epidemiological geographic scale. To accomplish this, we compared spoligotyping and RFLP results obtained in Italy and those on strains showing the same spoligotyping genotype found in other geographical contexts.

Indeed, the comparison of results among our Italian and other spoligotyping databases to monitor the behaviours of different spoligotypes was problematic. In our opinion, as each geographical region, nation or continent has its own predominant characteristic spoligotyping patterns due to a specific and different phylogenetic development of some tuberculous strains typical of each geographic area, no comparison or transposition of data can be made between different contexts regarding the probability values calculated for a specific studied population. In fact, the comparison between our cohort and other data collected around the world confirmed this hypothesis, showing very different probability values when the same strains were analyzed in different national contexts, suggesting that the comparison among spoligotyping patterns from different

countries should not be performed with widely epidemiological purposes and for the analysis of epidemiological links. Nevertheless, we still think that a wide spoligotyping based study could be highly useful as for population genetics, for taxonomical and evolutionary studies as well as for phylogeographical researches [25,26].

In conclusion, we found that the usefulness in clinical practice of the conditional probability value assigned to each spoligotype is poor in general, and limited to the geographical area in which the study has been conducted. Thus, we think that a world-wide spoligotyping database could be of interest for the evolutionary population genetics studies but we are also cautious in considering its real clinical usefulness. Instead, we recommend the use of spoligotyping as the most efficacious genotyping technique when tuberculosis outbreaks are suspected or confirmed, and to monitor tuberculosis transmission within a restricted geographic area or institutional setting.

Acknowledgements

We thank the staff of the Department of Infectious Diseases, 'L. Sacco' Hospital and of the Institute of Infectious Disease of the University of Milan, including Cecilia Paoli, and Patrizia Franza. We are indebted to Alan Michael Rosen, Dr Ned Rosen and Beth Sheidley for their assistance in the English revision of the, and to Annalisa Angelantoni of the AHSI Company for her continuous support.

This work was performed with the contribution of EU Concerted Action Project on 'New generation genetic markers and techniques for the epidemiology and control of tuberculosis' (QLK2-CT-2000-00630).

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